Isothiocyanate induction of apoptosis in cells overexpressing Bcl-2

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Kristin K. Brown

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Abstract

The oncogenic protein Bcl-2 is overexpressed in many cancers and prevents cells from undergoing apoptosis in response to traditional chemotherapeutic agents. Recent research has focussed on the development of novel agents that can disrupt the function of Bcl-2 and trigger apoptosis in cancer cells. The isothiocyanates are a class of naturally-occurring phytochemical with potential for development as both chemopreventive and chemotherapeutic agents. This thesis investigated the ability of the isothiocyanates to induce apoptosis in cells that overexpressed Bcl-2.

Initially, phenethyl isothiocyanate was shown to be cytotoxic to the Jurkat T-lymphoma cell line with an LD_{50} of 7.4 μM. Bcl-2 expression had little protective effect, and even greater than 50-fold overexpression only increased the LD_{50} to 15.1 μM. Morphological and biochemical assays indicated that death still occurred by apoptosis despite overexpression of Bcl-2.

A variety of other isothiocyanates were also screened for cytotoxic activity. While the isothiocyanate moiety was crucial for induction of apoptosis, the chemistry of the side chain attached to the isothiocyanate moiety also profoundly influenced the ability of an isothiocyanate to kill Bcl-2 overexpressing cells. The aromatic isothiocyanates were generally far more cytotoxic than aliphatic isothiocyanates. However, within the aromatic isothiocyanates tested in this study the length of the carbon linker group, between the phenyl ring and the isothiocyanate moiety, also influenced cytotoxic activity. Phenethyl isothiocyanate was identified as the most promising compound when targeting cells that overexpressed Bcl-2. Given that minor structural alterations significantly altered cytotoxic activity it is hypothesised that specific interactions with cellular targets may mediate induction of apoptosis by the isothiocyanates.

Finally, using a sensitive proteomic technique to label oxidised thiol proteins a preliminary investigation of the targets of the isothiocyanates was performed. A number of thiol proteins were selectively modified following exposure to phenethyl

isothiocyanate. One thiol protein that consistently changed was identified as mitochondrial peroxiredoxin-3. Changes to the oxidation state of peroxiredoxin-3 occurred well before activation of apoptosis and may play a role in mediating induction of apoptosis in cells that overexpress Bcl-2.

The results of this thesis have provided a platform to permit further investigation of the chemotherapeutic potential of the isothiocyanates and investigation of the mechanisms that allow the isothiocyanates to induce apoptosis in cells that overexpress the oncogene Bcl-2. In the future, the identification of primary targets of the isothiocyanates may aid the design and testing of novel anticancer drugs, and it will also provide novel insight into the regulation of apoptosis.

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Abbreviations

2-D	two-dimensional
Ac-DEVD-AMC	*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin
ADP	adenosine diphosphate
$AgNO_3$	silver nitrate
AITC	allyl isothiocyanate
AMC	amino-4-methylcoumarin
ANOVA	analysis of variance
Apaf-1	apoptotic protease activating factor-1
APS	ammonium persulfate
ARE	antioxidant responsive element
ATP	adenosine triphosphate
Bak	Bcl-2-antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphocyte/leukemia-2
Bcl-X_L	Bcl-2-like 1 isoform
BH	Bcl-2 homology
Bid	BH3-interacting domain death agonist
BITC	benzyl isothiocyanate
BOP	*N*-nitrosobis(2-oxopropyl)amine
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CHAPS	3[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid
CMV	cytomegalovirus
CO_2	carbon dioxide
cyt c	cytochrome c
DISC	death-inducing signalling complex
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNCB	1-chloro-2,4-dinitrobenzene
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGTA	ethyleneglycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FADD	Fas-associating protein with death domain
FasL	Fas ligand
FBS	fetal bovine serum
G_2/M	gap phase 2/mitosis
G-418	geneticin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
GST	glutathione *S*-transferase

H_2O_2	hydrogen peroxide
HCl	hydrochloride
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid
HRP	horse radish peroxidase
IAF	5-iodoacetamidofluorescein
IC_{50}	dose at which fifty percent of proliferation is inhibited
IEF	isoelectric focussing
IgG	immunoglobulin G
IPG	immobilized pH gradient
ITC	isothiocyanate
Keap1	Kelch-like ECH-associated protein 1
LD_{50}	dose at which fifty percent of cell viability is lost
MALDI-TOF	matrix assisted laser desorption/ionization time of flight
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MW	molecular weight
NAc	*N*-acetyl cysteine
NaCl	sodium chloride
NaOH	sodium hydroxide
NCS	isothiocyanate
NEM	*N*-ethyl maleimide
NF-κB	nuclear factor-kappa B
NNK	4-(methylnitrosamino)-1-butanone
NP-40	Nonidet P-40
Nrf-2	nuclear transcription factor erythroid 2p45-related factor 2
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PEITC	phenethyl isothiocyanate
pI	isoelectric point
PI	propidium iodide
PITC	phenyl isothiocyanate
polyA	poly-adenylate
Prx-3	peroxiredoxin-3
PVDF	polyvinyldene difluoride
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SFN	sulforaphane
SV40	simian virus 40
tBid	truncated Bid
TBS	Tris-buffered saline
$TBST_{20}$	Tris-buffered saline with 0.1% Tween™20
TEMED	N′,N′,N′,N′-tetramethylethylenediamine
TMB	tetramethyl-benzidine

Chapter 1

Introduction

The anti-apoptotic protein Bcl-2 is overexpressed in many cancers. Overexpression of this oncogene can abrogate the cytotoxic activities of chemotherapeutic agents resulting in multi-drug resistance. Current research is focussed on the identification of novel agents to overcome the anti-apoptotic block that overexpression of Bcl-2 confers. This thesis investigates the potential of a class of phytochemicals, called isothiocyanates, to induce apoptosis in cells that overexpress Bcl-2.

1.1 The isothiocyanates

Epidemiological studies demonstrate a clear relationship between increased consumption of fruits and vegetables and reduced occurrence of cancer (Steinmetz and Potter 1991; Steinmetz and Potter 1996). In particular, increased consumption of cruciferous vegetables from the *Brassica* genus has been correlated with a decrease in the incidence of various cancers (Spitz et al. 2000; Seow et al. 2002; Fowke et al. 2003; Bianchini and Vainio 2004). Commonly consumed Brassica vegetables include cabbage, broccoli, Brussels sprouts and watercress. The chemopreventive activity of these vegetables is largely attributed to amino-acid derived secondary products called glucosinolates, which are β-thioglucoside *N*-hydroxysulfates with a variable side chain and a sulfur-linked β-D-glucopyranose moiety (Holst and Williamson 2004) (Figure 1.1).

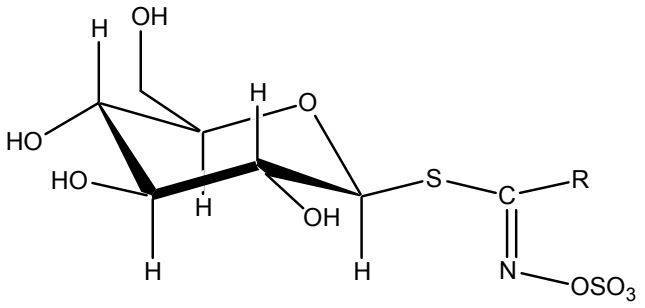


Figure 1.1: Chemical structure of the glucosinolates. *Glucosinolates exist as* N-*hydroxysulfates with a sulfur-linked β-glucose and variable side chain (R) (adapted from Fahey et al. 2001).*

More than 120 naturally occurring glucosinolates have been reported (Fahey et al. 2001). As many as fifteen different glucosinolates can be found in the same plant, although usually only three or four predominate (Holst and Williamson 2004). Watercress (*Nasturtium officinale*) contains eight glucosinolates, including glucoibarin, glucohirsutin, glucotropaeolin, glucosinalbin and, predominantly, gluconasturtiin (Daxenbichler et al. 1991; Fahey et al. 2001). On the other hand, the glucosinolate content of broccoli (*Brassica oleracea*) consists almost entirely of glucoraphanin, glucoerucin and glucoiberin (Fahey et al. 1997).

Although the glucosinolates are relatively biologically inert, they are converted into biologically active isothiocyanates by β-thioglucosidases called myrosinases (Bones and Rossiter 1996). In intact plant tissue, the myrosinases are localised in 'myrosin cells' and are physically separated from glucosinolates, which are located within the vacuole of a plant cell (Halkier and Du 1997). When the tissues of such plants are damaged, the glucosinolates come into contact with myrosinase, resulting in the conversion of the glucosinolate to a highly unstable aglycone which, in turn, undergoes spontaneous Lossen rearrangement to form the corresponding isothiocyanate (Chen and Andreasson 2001; Conaway et al. 2002) (Figure 1.2). Given that cruciferous vegetables are often consumed after being cooked, plant-derived myrosinase activity is usually absent; however, the conversion of glucosinolate to isothiocyanate can occur in the digestive system, as myrosinases are also present in many bacteria that are commonly associated with human gut microflora (Getahun and Chung 1999).

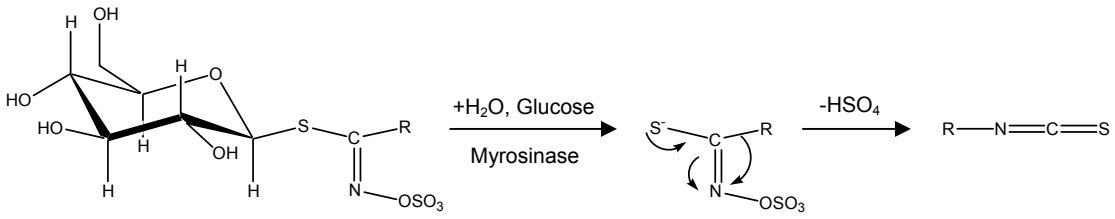


Figure 1.2: Formation of an isothiocyanate by hydrolysis of a glucosinolate *(adapted from Chen and Andreasson 2001)****.*** *Isothiocyanates are produced as a result of myrosinase-dependent hydrolysis of glucosinolates. An unstable aglycone (a thiohydroxamate-*O*-sulfonate) is the intermediate in the hydrolysis reaction.*

Within the plant itself, the isothiocyanates are thought to function as a defence against herbivore attack; however, these compounds also demonstrate antibacterial and antifungal activity (Manici et al. 1997; Lin et al. 2000; Mithen et al. 2000). More recently, the isothiocyanates have attracted much attention for their considerable potential as chemopreventive and chemotherapeutic agents.

1.1.1 Metabolism of the isothiocyanates

Oral administration of isothiocyanates has been shown to lead to rapid cellular accumulation in all organs of the body (Conaway et al. 1999; Conaway et al. 2002). Once ingested, isothiocyanates rapidly accumulate within cells and intracellular concentrations of these phytochemicals can reach millimolar levels (Zhang and Talalay 1998). Initially, isothiocyanates cross the plasma membrane by passive diffusion (Figure 1.3). Once inside the cell, the enzymatic actions of glutathione *S*-transferases (GSTs) catalyse the rapid conjugation of isothiocyanates with glutathione (GSH) to produce a dithiocarbamate (Zhang et al. 1995; Zhang 2001). Intracellular dithiocarbamates are rapidly depleted by expulsion through both the multidrug resistance associated protein-1 and P-glycoprotein-1 (Callaway et al. 2004). Upon leaving the cell, the dithiocarbamate can take one of two pathways. In the first pathway, the dithiocarbamate is converted back to the parent isothiocyanate by removal of GSH. This pathway regenerates the isothiocyanate, which can once again enter a cell by passive diffusion (Bruggeman et al. 1986; Zhang and Callaway 2002). The second pathway involves the processing of the dithiocarbamate via the mercapturic acid pathway, which ultimately allows for urinary excretion of the *N*-acetyl cysteine derivative of the dithiocarbamate (Ji and Morris 2003; Vermeulen et al. 2003).

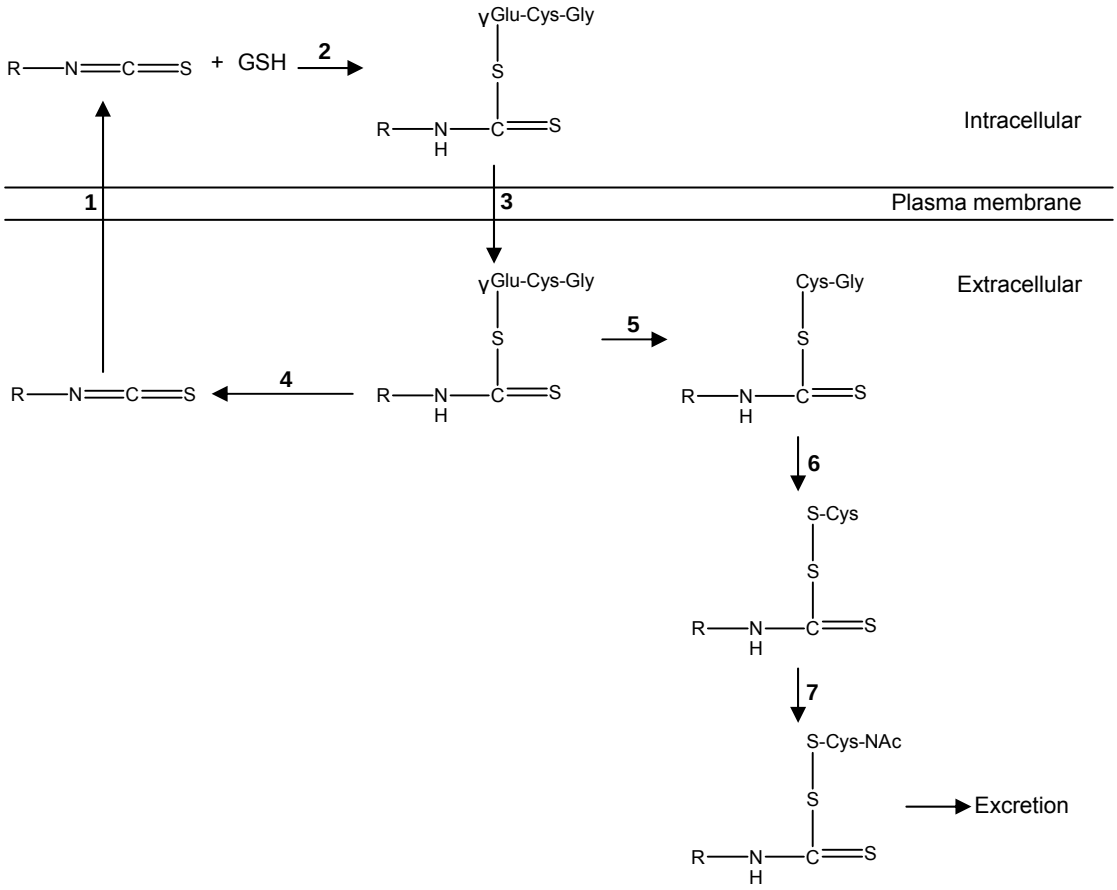


Figure 1.3: Metabolism of isothiocyanates *(adapted from Keum et al. 2004).* ***(1)*** *Extracellular isothiocyanates cross the cell membrane by passive diffusion.* ***(2)*** *GSTs catalyse the rapid conjugation of intracellular isothiocyanates with GSH.* ***(3)*** *The resulting dithiocarbamate is exported from the cell where* ***(4)*** *the parent isothiocyanate can be regenerated. Alternatively, the dithiocarbamate may be metabolised via the mercapturic acid pathway. Initially,* ***(5)*** *γ-glutamyl-transpeptidase converts the dithiocarbamate to a cysteinyl glycine derivative.* ***(6)*** *Cysteinyl-glycinase then converts the cysteinyl glycine derivative to a cysteine derivative. Finally,* ***(7)*** N*-acetyltransferase converts the cysteine derivative to an* N*-acetylcysteine derivative, which can be excreted in the urine.*

1.1.2 Intracellular reactions of the isothiocyanates

The chemistry of the isothiocyanate moiety will mediate the majority, if not all, of the physiological effects induced in response to isothiocyanate exposure. The central carbon of the isothiocyanate moiety is often highly electrophilic and can react with two cellular nucleophilic targets: amines and thiols (Thornalley 2002) (Figure 1.4). It is widely believed that the protective activities of the isothiocyanates may be mediated primarily via the reaction of the central carbon atom with these targets (Zhang et al. 2005).

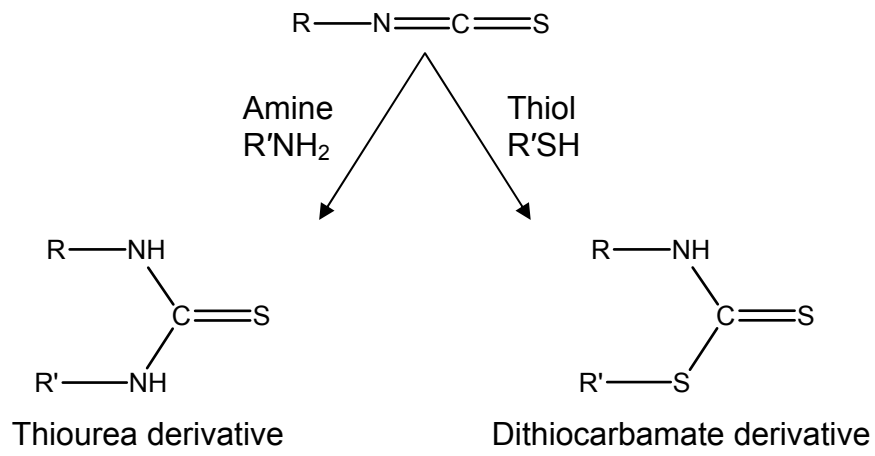


Figure 1.4: Key reactions of isothiocyanates with cellular targets*. Upon entering a cell an isothiocyanate can react with amines or thiol groups to produce thiourea or dithiocarbamate derivatives respectively (adapted from Thornalley 2002).*

The reaction of an isothiocyanate with an amine group results in the formation of a thiourea derivative. The modification of amines by isothiocyanates is utilised, in combination with mass spectrometry or high performance liquid chromatography (HPLC), for the sequencing of proteins and peptides (Muramoto et al. 1984; Gu and Prestwich 1997; Mo et al. 1997; Liu and Liang 2001). While the reaction of an isothiocyanate with an amine group is favoured above pH 9.5 (Maeda et al. 1969; Hernandez-Triana et al. 1996), at physiological pH, the predominant reaction is the conjugation of an isothiocyanate to a thiol, giving rise to a dithiocarbamate derivative (Podhradsky et al. 1979; Zhang et al. 2006). As previously discussed (section 1.1.1), this reaction occurs between an isothiocyanate and GSH. The formation of isothiocyanate-GSH adducts depletes the cell of its primary redox buffer and this has the potential to disrupt the function of sensitive thiol proteins in the cell (Xu and Thornalley 2001a). However, the direct modification of cysteinyl thiol groups, to form protein dithiocarbamates, is also likely to constitute an important component of isothiocyanate action.

While the isothiocyanate moiety itself is central to the biological actions of the isothiocyanates, the side chain is likely to influence biological activity. Numerous studies have already demonstrated the differing potencies of diverse isothiocyanates (Xu and Thornalley 2000b; Tang and Zhang 2004; Jakubikova et al. 2005). It is widely believed that the side chains of the isothiocyanates may influence factors such as the electrophilicity of the –N=C=S group, altering the steric hindrance to the

central carbon atom and controlling the lipophilicity of the molecule (Zhang et al. 2005).

1.1.3 Isothiocyanates and cancer prevention

The chemopreventive potential of the isothiocyanates has been attributed to several different actions (Hecht 2000; Zhang et al. 2006). The isothiocyanates are strong inhibitors of phase I enzymes, particularly the cytochrome P_{450} enzymes (Goosen et al. 2000; Nakajima et al. 2001), which convert environmental carcinogens into a much more toxic form. The isothiocyanates can inhibit cytochrome P_{450} activity by competitive, non-competitive and mechanism-based inhibition (Moreno et al. 1999; Goosen et al. 2001; Nakajima et al. 2001; von Weymarn et al. 2005). Such inactivation is likely to explain the results obtained in early rodent studies, where isothiocyanates could block carcinogenicity of compounds such as *N*-nitrosobis(2-oxopropyl)amine (BOP) and 4-(methylnitrosamino)-1-butanone (NNK), which require conversion to their active forms by cytochrome P_{450} enzymes (Smith et al. 1993; Hecht et al. 1996; Nishikawa et al. 1997).

Another important activity of the isothiocyanates is induction of phase II detoxification enzymes. The phase II enzyme family consists of a diverse range of enzymes including sulfotransferases, NAD(P)H:quinone oxidoreductases, and *N*-acetyltransferases (Xu et al. 2005a). Generally, phase II enzymes catalyse the conjugation of carcinogens with endogenous ligands, resulting in the formation of hydrophilic conjugates, which are often less toxic and more easily excreted in the urine or bile (Holtzclaw et al. 2004). The isothiocyanates activate phase II enzymes and consequently reduce carcinogen titre within the body (Rose et al. 2000). Activation of phase II enzymes by isothiocyanates also confers protection against oxidative insult via the induction of GST and thioredoxin reductase, two key enzymes involved in the maintenance of thiol homeostasis (Gao et al. 2001; Ye and Zhang 2001; Hintze et al. 2003).

The induction of phase II enzymes by isothiocyanates is primarily mediated by activation of the transcription factor Nrf2 (nuclear transcription factor erythroid 2p45-related factor 2) (Ye and Zhang 2001). Normally, Nrf2 is anchored in the cytoplasm by Keap1 (Kelch-like ECH-associated protein 1). Under conditions of stress, or

following isothiocyanate exposure, Nrf2 dissociates from Keap1 (Itoh et al. 1999) and translocates to the nucleus where it can proceed to bind the DNA regulatory antioxidant responsive elements (AREs) to induce transcription of phase II enzymes (Itoh et al. 1997; Lee and Surh 2005). Oxidation, or covalent modification, of cysteine residues within Keap1 is sufficient to disrupt the Nrf2-Keap1 complex (Dinkova-Kostova et al. 2002; Hong et al. 2005b) (Figure 1.5). The isothiocyanate sulforaphane has been demonstrated to activate Nrf2 via the formation of covalent adducts with selected cysteine residues in Keap1 (Hong et al. 2005a).

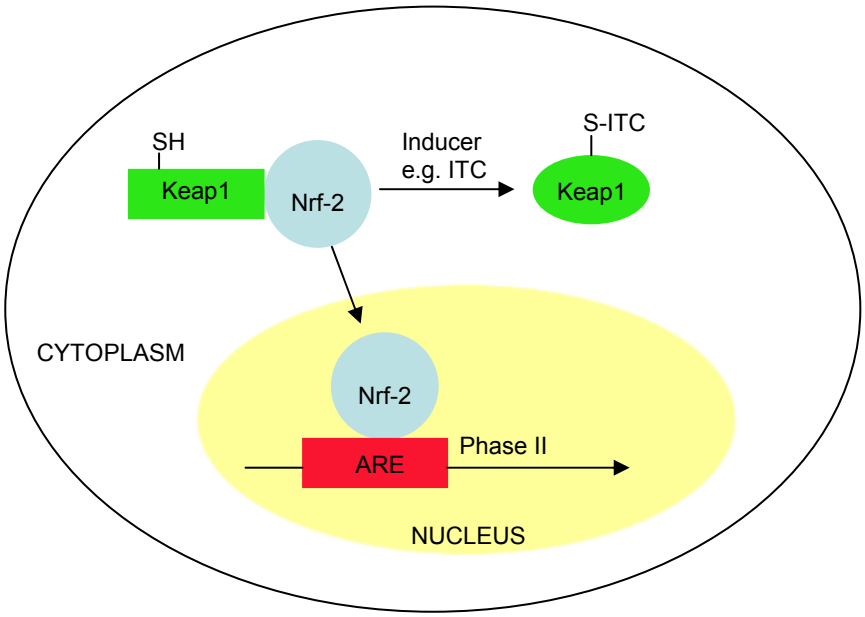


Figure 1.5: Regulation of phase II detoxification enzymes *(adapted from Dinkova-Kostova et al. 2002). The transcription factor Nrf2 is anchored in the cytoplasm by binding to Keap1. Inducers, including some isothiocyanates (ITC) disrupt the Keap1-Nrf2 complex, and Nrf2 migrates to the nucleus where it binds ARE regions of phase II genes and accelerates their transcription.*

The chemopreventive effects of the isothiocyanates were traditionally attributed to the above processes of enhancement of carcinogen detoxification by phase II induction and the blocking of carcinogen activation by phase I inhibition (Hecht 1999). Both of these actions explain the ability of the isothiocyanates to prevent tumourigenesis when administered prior to carcinogen exposure (Morse et al. 1991; Jiao et al. 1997; Kassie et al. 2002). However, the isothiocyanates have also been shown to inhibit tumour formation when administered after carcinogen insult (Chung et al. 2000; Yang et al. 2002). An alternate mechanism to explain the chemopreventive activity of the isothiocyanates was revealed when a selection of isothiocyanates were shown to

induce a form of cell death termed apoptosis (Yu et al. 1998). It has since been shown that a variety of isothiocyanates possess a potent ability to inhibit cell proliferation and induce apoptosis in a wide variety of cell types. The ability of compounds to trigger apoptosis in precancerous cells is currently being explored as an alternate mechanism of chemoprevention (Sun et al. 2004). In addition to the mechanisms described above, chemoprevention by the isothiocyanates has been linked to inhibition of the NF-κB (nuclear factor-kappa B) signal transduction pathway (Heiss et al. 2001; Jeong et al. 2004; Xu et al. 2005b), inhibition of histone deacetylase (Myzak et al. 2004; Myzak et al. 2006a; Myzak et al. 2006b), inhibition of free-radical production by phagocytic cells (Manesh and Kuttan 2003; Miyoshi et al. 2004) and inhibition of angiogenesis (Bertl et al. 2006). The focus of this thesis is the ability of the isothiocyanates to promote apoptosis.

1.2 Apoptosis

To protect tissue homeostasis, all dividing cell populations maintain a balance between cell production and cell loss. Most cell loss observed during normal turnover is attributed to a process known as apoptosis. Apoptosis can be defined as a form of programmed cell death that is either developmentally regulated, launched in response to specific stimuli or activated in response to various forms of injury or stress (Chandra et al. 2000; Kaufmann and Earnshaw 2000; Raghupathi et al. 2000; Twomey and McCarthy 2005).

The term apoptosis was first introduced following observations of a morphologically distinct type of cell death that occurred primarily under physiological conditions (Kerr 1971; Kerr et al. 1972). Apoptosis is characterised by the sequential appearance of chromatin margination and fragmentation, cellular shrinkage, and a budding fragmentation (Saraste and Pulkki 2000). The morphological features of an apoptotic cell result from the execution of a genetically controlled autodigestion pathway. The key event in this pathway is the activation of endogenous proteases called caspases (Cohen 1997; Chang and Yang 2000). Prior to the initiation of apoptosis, the caspases exist in a cell as inactive proenzymes. When the apoptotic pathway is initiated, procaspases are processed by cleavage at internal aspartate residues to form active caspases (Earnshaw et al. 1999). The active caspases then proceed to cleave specific

protein substrates to bring about the controlled degradation of a cell (Fischer et al. 2003).

1.2.1 Apoptotic signalling pathways

Two distinct, but interconnected, pathways of caspase activation have been characterised: the cell surface death receptor (extrinsic) pathway and the mitochondria-initiated (intrinsic) pathway.

The extrinsic caspase activation pathway is triggered by binding of cell surface death receptors with their specific ligands in response to an extracellular pro-apoptotic stimulus. Ligand binding induces receptor oligomerization and the recruitment of intracellular receptor-associated proteins. The best characterised member of the death receptor superfamily is Fas (Figure 1.6A). Binding of the Fas ligand to the Fas receptor induces oligomerization of Fas and formation of the death-inducing signalling complex (DISC) (Peter and Krammer 2003). The cytoplasmic region of the DISC contains a death domain that binds the adaptor molecule FADD (Fas-associating protein with death domain), which, in turn, recruits procaspase-8 (Kaufmanna et al. 2002). The association of procaspase-8 with FADD enables auto-proteolytic processing to generate active caspase-8 (Nicholson and Thornberry 1997). Caspase-8 in turn activates caspase-3, the key executioner of apoptosis responsible for the proteolytic cleavage of cellular proteins (Fischer et al. 2003).

The extrinsic signalling pathway diverges following formation of the DISC (Scaffidi et al. 1998). In Type I cells, where the activation of procaspase-8 is vigorous, active caspase-8 can directly cleave and activate procaspase-3. In contrast, Type II cells are characterised by insufficient activation of caspase-8 and subsequent failure to activate procaspase-3. Therefore, in Type II cells, apoptosis is dependent on caspase-8 cleaving the cytosolic Bcl-2 protein Bid to generate a truncated fragment (tBid) (Kaufmann and Hengartner 2001). tBid acts at the mitochondria to indirectly activate the intrinsic pathway (Li et al. 1998) (Figure 1.6B). This mechanism allows for a significant degree of crosstalk between the two pathways and also for amplification of the apoptotic response.

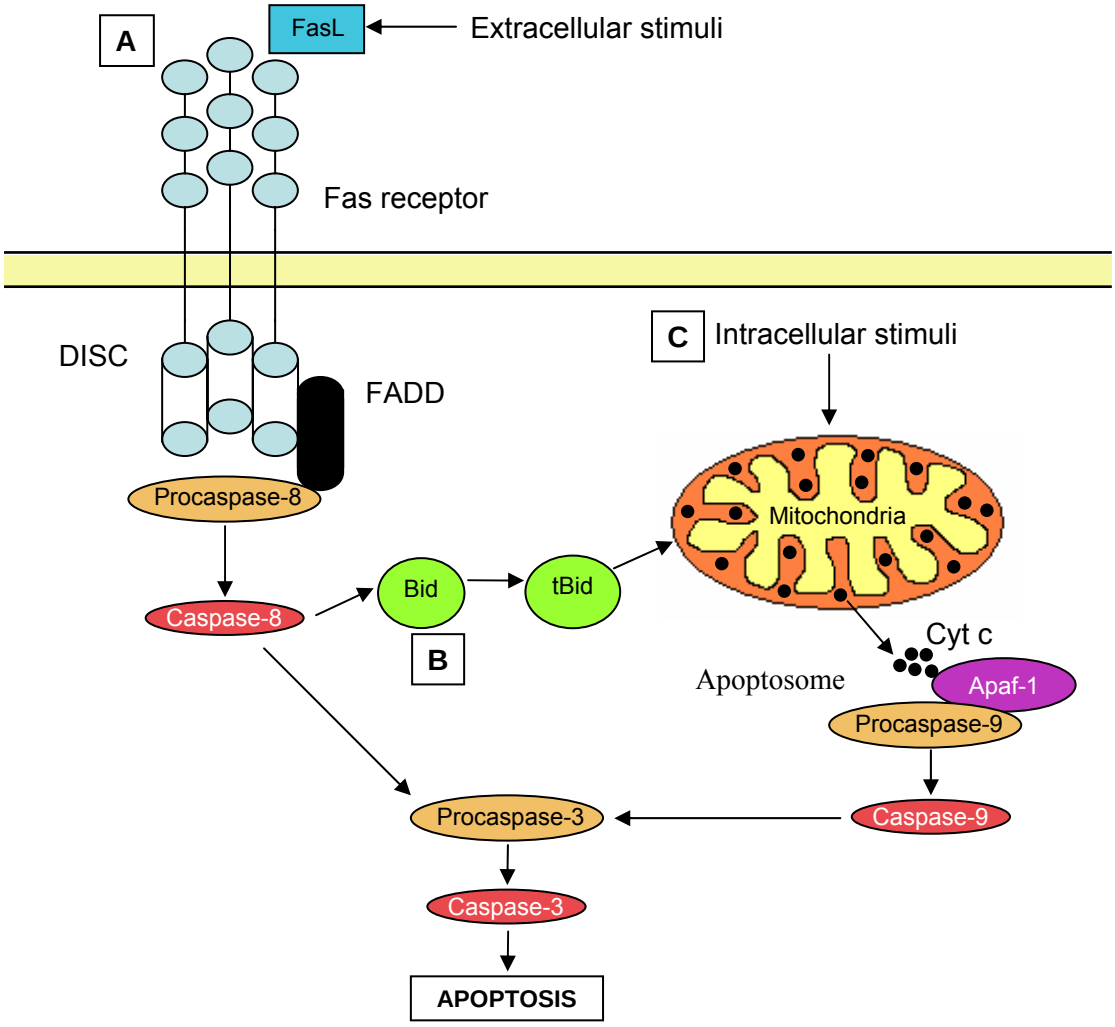


Figure 1.6: Pathways that lead to caspase activation and apoptosis *(reviewed by Green 1998).* ***(A)*** *The extrinsic pathway is initiated when an extracellular ligand binds to a death receptor. Ligand binding induces the oligomerization of the death receptor, which in turn allows for the recruitment of adaptor proteins that contain a binding site for procaspase-8. The formation of such a complex induces the auto-proteolytic activation of the inactive procaspase to active caspase-8. Caspase-8 then proceeds to activate caspase-3.* ***(B)*** *The extrinsic pathway can also initiate or amplify apoptosis via cleavage of the pro-apoptotic factor Bid by caspase-8. The truncated fragment (tBid) then acts at the mitochondria to induce the release of cytochrome c (Cyt c).* ***(C)*** *The intrinsic pathway is initiated by the release of pro-apoptotic factors including cytochrome c from the mitochondria in response to an intracellular stimulus. The binding of cytosolic cytochrome c to Apaf-1 triggers the recruitment of procaspase-9 via a caspase recruitment domain. Procaspase-9 is then activated to caspase-9 and in turn proceeds to activate caspase-3.*

As well as being triggered via activation of the extrinsic pathway, the intrinsic caspase activation pathway is induced in response to intracellular apoptotic stimuli, including hypoxic stress, chemotherapeutic agents and growth factor withdrawal (Kim and Park 2003; Bian et al. 2004; Cornelis et al. 2005). The conversion of an intracellular

stimulus into initiation of the intrinsic apoptotic cascade relies on alterations at the mitochondria. The critical step in the initiation of the intrinsic pathway is the release of cytochrome c from where it normally resides, in the intermembrane space of mitochondria, to the cytosol, where it can complex with Apaf-1 (Liu et al. 1996) (Figure 1.6C). Apaf-1 will initially bind ATP and hydrolyse it to ADP. This process facilitates the formation of a multimeric Apaf-1/cytochrome c complex called the apoptosome, which can then recruit and subsequently activate procaspase-9 (Zou et al. 1999; Cain 2003). The intrinsic caspase activation pathway culminates in caspase-9-mediated activation of the executioner caspase, caspase-3 (Kaufmann and Hengartner 2001). The activation of the intrinsic caspase activation pathway is centred on the regulation of cytochrome c release from the mitochondria, which is mediated by the Bcl-2 family of pro- and anti-apoptotic proteins (Kluck et al. 1997; Cai et al. 1998; Scorrano and Korsmeyer 2003).

1.3 The Bcl-2 family of pro- and anti-apoptotic proteins

The Bcl-2 family plays a critical role in whether or not a cell will undergo apoptosis. Over twenty-five Bcl-2 family members have been identified, and these can be broadly divided into three functional groups (Adams and Cory 1998). Members of the first group, which include Bcl-2 and Bcl-X_L, are characterized by the presence of four Bcl-2 homology (BH) domains (BH1-BH4). All members of this group display anti-apoptotic activity (Huang et al. 1998). Members of group two, including Bax and Bak, have a similar overall structure to the group one proteins but lack the BH4 domain and display pro-apoptotic activity. Group three covers a diverse collection of pro-apoptotic proteins, including Bid, whose only common feature is the presence of the BH3 domain. The balance between the expression and activity of pro-apoptotic and anti-apoptotic members of the Bcl-2 family provides a critical switch in the determination of whether or not a cell will undergo apoptosis (Mirjolet et al. 2000; Borner 2003).

1.3.1 Bcl-2

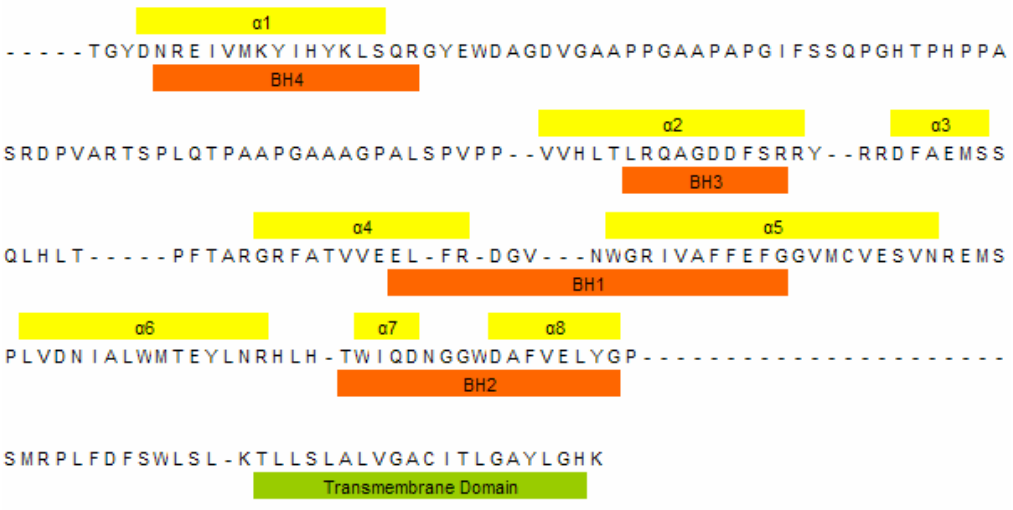
The founding member of the Bcl-2 family of proteins was the B-cell lymphocyte/leukemia-2 (Bcl-2) oncoprotein. Bcl-2 was initially identified at the breakpoint of the t(14;18) chromosomal translocation that occurs in a large majority of patients with non-Hodgkin's B-cell lymphoma (Tsujimoto et al. 1984). As a result

of this translocation, the expression of bcl-2 comes under the control of the immunoglobulin heavy chain enhancer and is therefore constitutively expressed in B cells (Graninger et al. 1987; Reed et al. 1987; Seto et al. 1988). Subsequent studies demonstrated that deregulation of Bcl-2 markedly prolongs the survival of mature B cells, resulting in an immortalized cell population (Vaux et al. 1988; McDonnell et al. 1989). The biochemical function of Bcl-2 was eventually elucidated in 1990, when it was discovered that deregulation of Bcl-2 prevents cell death by blocking apoptosis (Hockenbery et al. 1990), although how Bcl-2 achieves this block continues to be a matter of controversy (Vander Heiden and Thompson 1999; Belka and Budach 2002; Annis et al. 2004).

1.3.2 Structure of Bcl-2

Bcl-2 is composed of eight α-helices that are connected by loops of varying length (Petros et al. 2001; Petros et al. 2004) (Figure 1.7A and B). Two central helices (α5 and α6) form the core of the protein. The central helices are flanked by helices α3 and α4 on one side and, on the other side, by helices α1 and α2. A largely unstructured loop exists between α1 and α2. The BH1, BH2 and BH3 domains of Bcl-2 are proximal to one another and form the top of an elongated hydrophobic groove, which is required for anti-apoptotic activity (Figure 1.7C). The bottom of this groove is formed by helices α3 and α4. The carboxy-terminal transmembrane domain is critical for the localization of Bcl-2 to the endoplasmic reticulum, nuclear envelope and the outer mitochondrial membrane (Krajewski et al. 1993). However, removal of the transmembrane domain only partially reduces the anti-apoptotic activity of Bcl-2, suggesting that membrane localisation is not absolutely essential for the function of Bcl-2 (Hunter et al. 1996).

A



B **C**

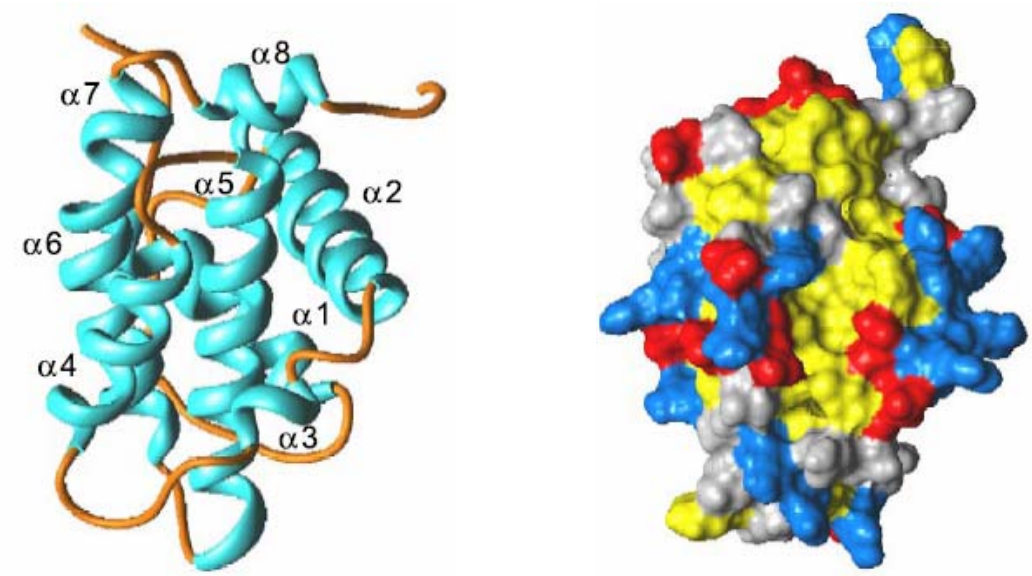


Figure 1.7: Structure of the anti-apoptotic protein Bcl-2. ***(A)*** *Primary structure of Bcl-2 highlighting the Bcl-2 homology (BH) domains the transmembrane domain and the positions of the α-helices (adapted from Petros et al. 2004).* ***(B)*** *Ribbon representation of the solution structure of Bcl-2 (Petros et al. 2004).* ***(C)*** *The Connolly surface model for Bcl-2 is coloured as follows: hydrophobic residues are yellow, basic residues are blue and acidic residues are red (Petros et al. 2004).*

1.3.3 Inhibition of apoptosis by Bcl-2

The exact mechanisms by which Bcl-2 can prevent apoptosis are still highly controversial; however, it is widely accepted that mitochondrial anchored Bcl-2 can maintain the integrity of the mitochondrial outer membrane and thereby prevent the

release of pro-apoptotic factors. Given the importance of cytochrome c release in the induction of apoptosis, a large collection of data has been presented to support the hypothesis that the release of cytochrome c from mitochondria is regulated, either directly or indirectly, by Bcl-2 and that overexpression of Bcl-2 can prevent the release of such pro-apoptotic factors (Kluck et al. 1997; Yang et al. 1997).

While Bcl-2 can prevent the release of cytochrome c, the pro-apoptotic Bcl-2 family member Bax (Bcl-2-associated X protein) can induce the release of cytochrome c from mitochondria (Jurgensmeier et al. 1998). Bax was initially identified as a heterodimerization partner of Bcl-2 and the ratio of Bcl-2 to Bax has been shown to determine the rate of apoptosis (Oltvai et al. 1993). Bax can form pores in the mitochondrial outer membrane which permit the release of pro-apoptotic factors from the mitochondrial intermembrane space. The ability of Bcl-2 to heterodimerize with Bax is thought to constitute a key mechanism of the anti-apoptotic function of Bcl-2. The hydrophobic groove formed by the BH1-3 regions of anti-apoptotic Bcl-2 family proteins mediates the interaction with the amphipathic, α-helical BH3 domain of pro-apoptotic counterparts such as Bax (Sattler et al. 1997) (Figure 1.7). Mutations in the hydrophobic region of Bcl-2 have been shown to block heterodimerization with other family members and, furthermore, abolish the anti-apoptotic activity of Bcl-2 (Yin et al. 1994; Minn et al. 1999). However, the sequestration of pro-apoptotic family members is likely to be just one of the mechanisms by which Bcl-2 prevents the release of pro-apoptotic factors from the mitochondria (Zamzami et al. 1998).

Another potential explanation for the anti-apoptotic activity of Bcl-2 arose when it was observed that Bcl-2 knockout mice display pathologies that are associated with defects in antioxidant pathways (Veis et al. 1993). This suggested that Bcl-2 plays a role in preventing cell death in response to oxidative stress. Further studies examining the enforced expression of Bcl-2 clearly demonstrated the ability of Bcl-2 to inhibit hydrogen peroxide-induced apoptosis (Hockenbery et al. 1993). The potential antioxidant function of Bcl-2 may be explained by the observation that an elevation in the cellular levels of Bcl-2 corresponds with a concomitant increase in intracellular levels of GSH (Mirkovic et al. 1997). Given that GSH acts as the primary reducing equivalent that can buffer and remove free radicals, an increase in GSH would confer resistance to oxidative stress-induced apoptosis (Kane et al. 1993; Ellerby et al. 1996;

Devadas et al. 2003). This mechanism was broadened to include non-oxidative stimuli, particularly because alterations in the redox environment of a cell are proposed to be a common feature of the apoptosis pathway; however, this remains a controversial area (Steinman 1995; Voehringer and Meyn 1998; Ueda et al. 2002).

1.4 Apoptosis resistance and cancer

Defects in the apoptotic pathway play an important role in the pathogenesis and progression of many cancers (Hanahan and Weinberg 2000). Defective apoptosis is necessary for cellular transformation, enabling the survival of damaged and mutated cells, and balancing the action of oncogenes that jointly enhance proliferation and apoptosis (Green and Evan 2002). In addition, defective apoptosis pathways contribute to multi-drug resistance (Johnstone et al. 2002). Many cancer cells possess or acquire the ability to resist chemotherapeutic agents, making the successful treatment of cancer very difficult.

A landmark study investigating the mechanism of action of several chemotherapeutic agents revealed that DNA fragmentation is induced in response to chemotherapy (Kaufmann 1989). Given that DNA fragmentation is one of the first features of a cell undergoing apoptosis, it was concluded that some chemotherapeutic agents induce apoptosis. It is now understood that a chemotherapeutic agent does not directly kill a cell but will induce a series of cellular responses that impact on tumour cell proliferation and survival (Lowe and Lin 2000). Probably the most important response of a cell to a chemotherapeutic agent is the induction of apoptosis. Due to the fact that diverse chemotherapeutic agents all act via initiation of the apoptotic pathway, small discrepancies in either the activation or execution phases of apoptosis can result in a multi-drug resistant phenotype (Ferreira et al. 2002).

1.4.1 Deregulation of Bcl-2 in cancer

In many forms of cancer, an imbalance between pro- and anti-apoptotic proteins leads to accumulation of cells and the inability to respond correctly to apoptotic stimuli. In fact, deregulation of such proteins strongly correlates with the incidence of certain types of cancer and also with the ability of a cancer patient to enter remission (Schimmer et al. 2003; Yang et al. 2003). Besides being overexpressed in a large majority of patients with non-Hodgkin's B cell lymphoma, the anti-apoptotic protein

Bcl-2 has been shown to be upregulated in a wide variety of human cancers including 80% of B-cell lymphomas, 30-60% of prostate cancers, and 90% of colorectal carcinomas (Kitada et al. 2003). The overexpression of Bcl-2 has serious consequences for cancer chemotherapy and can abrogate the cytotoxic activities of a wide variety of standard chemotherapeutic agents by blocking chemotherapy-induced apoptosis (Campos et al. 1993; Miyashita and Reed 1993; Park et al. 2001; Kim et al. 2003).

Direct inhibition or downregulation of Bcl-2 has been shown to be lethal to cancer cells and is, therefore, a valid target for cancer therapy (Strasser et al. 1990; Letai et al. 2004; Letai 2005). Three key strategies have been employed to target Bcl-2. The first strategy involves the development of antisense molecules which degrade mRNA and, therefore, downregulate the levels of Bcl-2 (Rudin et al. 2004). The other two strategies employ molecules that bind the hydrophobic pocket of Bcl-2 and prevent the interaction of the anti-apoptotic protein with pro-apoptotic counterparts. Such inhibition sensitizes cells to the effects of death signals. The first of these Bcl-2-binding strategies utilises BH3-domain peptides from pro-apoptotic Bcl-2 proteins including Bax (Finnegan et al. 2001). The second Bcl-2 binding strategy employs small-molecule inhibitors of Bcl-2 (Oltersdorf et al. 2005; Milanesi et al. 2006). Recent efforts have focussed on the identification and design of small organic molecules that are capable of antagonizing Bcl-2 function. Such inhibitors are more advantageous than peptide-based drugs as they can be refined for optimal solubility, cell permeability, stability, binding affinity and activity (Shangary and Johnson 2003). One class of compounds receiving interest as novel cancer therapeutics targeting Bcl-2 are the naturally occurring plant polyphenols that can bind the BH3 binding pocket of anti-apoptotic Bcl-2 proteins with extreme efficiency (Kitada et al. 2003). In addition to the plant polyphenols, Pellecchia and Reed (2004) speculated that other plant phytochemicals with anticancer properties, including the isothiocyanates, may act in a similar way (Pellecchia and Reed 2004).

1.5 Ability of isothiocyanates to trigger apoptosis in cells overexpressing Bcl-2

Previous work from our laboratory explored the possibility that elevated GSH levels played a role in the resistance of Bcl-2 expressing cells to Fas-mediated apoptosis. This hypothesis was subsequently disproved when agents that depleted GSH were

unable to sensitize the cells to apoptosis. However, one of the compounds used, phenethyl isothiocyanate, showed a dramatic ability to sensitize the cells to apoptosis (Pullar et al. 2004). Subsequent experiments were undertaken to determine if phenethyl isothiocyanate could also sensitize the resistant cells to chemotherapeutic drugs. However, these preliminary studies suggested that phenethyl isothiocyanate was able to directly trigger apoptosis in the cells overexpressing Bcl-2.

The aim of this thesis was to directly examine this phenomenon by:

1. An investigation of the ability of phenethyl isothiocyanate to induce apoptosis in cells overexpressing Bcl-2. The anti-proliferative activity, cytotoxic activity and apoptosis-inducing potential of phenethyl isothiocyanate was examined in sensitive cells and cells overexpressing varying levels of Bcl-2.
2. A structure-activity investigation of a selection of isothiocyanates to determine the key structural features of the isothiocyanates that confer apoptosis-inducing properties in cells overexpressing Bcl-2. Sixteen isothiocyanates with diverse side chain chemistry were screened for anti-proliferative activity, cytotoxic activity and ability to sensitise cells to Fas-mediated apoptosis in sensitive cells and Bcl-2 overexpressing cells.
3. A preliminary investigation into the key cellular targets of the isothiocyanates that promote induction of apoptosis. A proteomic technique for labelling oxidised thiol proteins was utilised to examine the response of the entire cellular complement of thiol proteins to exposure to the isothiocyanates.

Chapter 2

Materials and Methods

2.1 Materials

Cell culture materials including RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin, and geneticin were purchased from Gibco BRL, Invitrogen (Auckland, New Zealand).

Hexyl isothiocyanate, *tert*-butyl isothiocyanate, 1-methyl allyl isothiocyanate and 1-methyl butyl isothiocyanate were provided by Dr Rex Munday, AgResearch (Ruakura, New Zealand). Allyl isothiocyanate was obtained from Sigma Chemical Co (St Louis, MO, USA). All other isothiocyanates were sourced from LKT Laboratories (St Paul, MN, USA).

The detergent compatible (D_C) protein assay kit, 40% acrylamide/bis solution 37.5:1 (2.6% C), Micro Bio-Spin® 6 chromatography columns, ReadyStrip 17cm, pH 3-10 IPG strips, Bio-Lyte® 3/10 ampholytes, mineral oil and electrode wicks were acquired from Bio-Rad Laboratories (Hercules, CA, USA).

DNA grade agarose was purchased from Progen Industries Ltd. (Darra, Australia)

Mouse anti-Bcl-2 and activating Fas antibodies were supplied by Zymed Laboratories (San Francisco, CA, USA).

Sheep anti-mouse IgG-horseradish peroxidase, Hybond-PVDF membrane and enhanced chemiluminescence (ECL™) Western blotting system were obtained from Amersham Biosciences (Buckinghamshire, England).

5-Iodoacetamidofluorescein (IAF) was sourced from Molecular Probes (Invitrogen, Auckland, New Zealand).

The cell proliferation ELISA, BrdU (colorimetric) kit, Complete™ protease inhibitors and 3[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) were acquired from Roche Diagnostics (Manheim, Germany).

The artificial caspase substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was acquired from the Peptide Institute Inc (Osaka, Japan).

All other chemicals and reagents were purchased from Sigma Chemical Co (St Louis, MO, USA) and BDH Laboratory Supplies (Poole, England).

All water was deionised and ultrafiltrated using a Milli-Q filtration system.

2.2 Solutions

2.2.1 Isothiocyanate Stock Solutions

The isothiocyanates were diluted with DMSO to make 100 mM stock solutions. Stock solutions were prepared using known MW and purity values (Table 2.1):

Isothiocyanate	**MW (g/mol)**	**Purity (%)**
Benzyl thiocyanate	149.21	99
Benzyl selenocyanate	196.11	99.24
Phenyl isothiocyanate	135.19	98
Benzyl isothiocyanate	149.22	98
Phenethyl isothiocyanate	163.24	99
3-Phenylpropyl isothiocyanate	177.27	99.2
4-Phenylbutyl isothiocyanate	191.30	100
Phenylhexyl isothiocyanate	219.35	98.81
R(-)-α-Methylbenzyl isothiocyanate	163.24	99.7
S-(*N*-benzylthiocarbamoyl)-*L*-cysteine	270.37	98
S-(*N*-3-phenylpropylthiocarbamoyl)-*L*-cysteine	298.42	99
t-Butyl isothiocyanate	115.20	100
Allyl isothiocyanate	99.16	95
1-Methyl allyl isothiocyanate	113.18	100
1-Methyl butyl isothiocyanate	129.23	100
Hexyl isothiocyanate	143.25	100
Sulforaphane	177.29	99.68

Table 2.1: Molecular weight and percentage purity of various isothiocyanates.

The appropriate mass of an isothiocyanate was weighed out and DMSO was added to bring the final volume to 1 mL. The stock solutions were stored at +4°C for up to two months.

Just prior to use, further dilutions of the isothiocyanate stock solutions were prepared in order that the final concentration of DMSO added to the cell culture medium was maintained at a constant 0.1%.

2.2.2 Cell viability analysis solutions

Propidium iodide

The propidium iodide staining solution was prepared by dissolving 5 mg of propidium iodide in 5 mL of milliQ water. Aliquots were stored in the dark at +4°C.

2.2.3 Caspase activity analysis solutions

Caspase buffer

The caspase buffer was made by combining:

- 100 mM HEPES
- 10% sucrose
- 0.1% CHAPS
- 0.1% NP-40

The solution was made to 100mL with milliQ water and the pH adjusted to 7.25 with NaOH. The caspase buffer was stored in aliquots at -20°C.

2.2.4 SDS-PAGE solutions

2 M Tris-HCl, pH 8.8

A 2 M Tris-HCl, pH 8.8 solution was prepared by dissolving 48 g of Tris base in 100 mL of milliQ water. The pH was adjusted to 8.8 before the solution was made to a final volume of 200 mL.

0.5 M Tris-HCl, pH 6.8

A 0.5 M Tris-HCl, pH 6.8 solution was prepared by dissolving 6 g of Tris base in 60 mL of milliQ water. The pH was adjusted to 6.8 before the solution was made to a final volume of 100 mL.

20% (w/v) SDS

A 20% solution of SDS was made by dissolving 20 g of SDS in 100 mL of milliQ water.

10% ammonium persulfate

A fresh 10% solution of ammonium persulphate (APS) was prepared just prior to gel preparation by dissolving 100 mg APS in 1 mL of milliQ water.

SDS-polyacrylamide gels

SDS-PAGE gels were prepared according to Table 2.2. Acrylamide, Tris-HCl and water were initially combined before addition of SDS and APS. The solution was thoroughly mixed before addition of TEMED.

Component	**15% gel for Western blotting (2 gels)**	**4% stacking gel for Western blotting (2 gels)**	**15% gel for 2-D electrophoresis (2 gels)**
40% acrylamide/bis solution 37.5:1	3.75 mL	500 µL	37.5 mL
2 M Tris/HCl, pH 8.8	2 mL	-	20 mL
0.5 M Tris/HCl, pH 6.8	-	1.25 mL	-
Water	4.15 mL	3.2 mL	41.5 mL
20% SDS	50 µL	25 µL	250 µL
10% APS	50 µL	25 µL	250 µL
TEMED	15 µL	8 µL	25 µL

Table 2.2: Components of SDS-PAGE gels for Western blotting and 2-D electrophoresis.

Sample loading buffer

Sample loading buffer was prepared by combining:

Distilled water	3 mL
0.5 M Tris-HCl, pH 6.8	1 mL
Glycerol	1.6 mL
10% SDS	1.6 mL
β-mercaptoethanol	0.4 mL
0.5% (w/v) bromophenol blue	0.4 mL

SDS-PAGE running buffer

The electrode running buffer was made by addition of 25 mM Tris, 192 mM glycine and 0.1% SDS to the appropriate volume of milliQ water.

2.2.5 Western Blotting solutions

Extract buffer

The extract buffer was made by combining milliQ water with: 40 mM HEPES
50 mM NaCl
1 mM EDTA
1 mM EGTA

The pH of the solution was adjusted to 7.4.

Sample extract buffer

To prepare the sample extract buffer, 40 µL of a Complete™ protease inhibitor solution and 40 µL of a 25% CHAPS solution were added to every 1 mL of extract buffer. The sample extract buffer was prepared just prior to use.

Western blot buffer

A Tris-glycine Western blot buffer was prepared by dissolving 25mM Tris and 192mM glycine in milliQ water with 10% methanol. The buffer was refrigerated for at least one hour before use.

10x Tris-buffered saline (TBS)

A 10x stock solution of TBS was made by addition of 24.2g Tris and 80g NaCl to 1 L of milliQ water. The pH of the solution was adjusted to 7.6.

$TBST_{20}$

A 1 L preparation of $TBST_{20}$ was prepared by combining 100 mL of 10x TBS and 500µL Tween™ 20 with 900 mL milliQ water.

2.2.6 Two-dimensional electrophoresis solutions

N-*ethyl maleimide (NEM) buffer*

NEM buffer was made by adding 0.0125 g of NEM and 40 µL of a protease inhibitor solution to every 1 mL of extract buffer (section 2.2.5). The NEM buffer was prepared just prior to use.

IAF solution

A 10 mM solution of IAF was prepared by dissolving 5.2 mg of IAF in 1 mL of DMSO. The IAF solution was stored in the dark at -20°C. A fresh preparation was made weekly.

Sample/rehydration solution

The sample/rehydration solution was prepared by combining:

7 M Urea
2 M Thiourea
10 mM DTT
4% CHAPS
0.2% Biolytes

A few crystals of bromophenol blue were added before the solution was made to a final volume of 40 mL by addition of milliQ water.

Equilibration buffer

The equilibration buffer was prepared by combining:

6 M Urea
0.375 M Tris
20% glycerol
2% SDS

The final volume was brought to 500 mL with milliQ water. A few crystals of bromophenol blue were added to the final solution.

0.5% agarose solution

The agarose solution was made by combining 2.5 g of agarose with 500 mL of 1x SDS-PAGE running buffer. The agarose was dissolved by heating in the microwave. A few crystals of bromophenol blue were added to the final solution. Just prior to use the agarose solution was heated in the microwave until molten.

2.2.7 Silver stain solutions

Solution 1

Silver stain solution 1 was made by dissolving 0.2 g of anhydrous sodium thiosulphate in 1 L of milliQ water.

Solution 2

Silver stain solution 2 was prepared by addition of 2 g silver $AgNO_3$ and 750 µL of formaldehyde to 1 L of milliQ water. A fresh preparation of solution 2 was made just prior to use.

Solution 3

Silver stain solution 2 was made by combining 60 g sodium carbonate, 20 mL of silver stain solution 1, 500 µL formaldehyde and 980 mL of milliQ water.

Solution 4

Silver stain solution 4 was prepared by dissolving 20 g of sodium EDTA in 1 L of milliQ water

2.3 Methods

2.3.1 Cell Culture

The Jurkat T lymphocyte cell line was obtained from American Type Culture Collection (Rockville, MD). Jurkat cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. The vector control and the Bcl-2 over-expressing lines were maintained in RPMI-1640 containing 10% FBS and 350 µg/ml geneticin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Cultured cells were collected at optimal growth (~0.8-1x10^6 cells/ml) and resuspended in fresh RPMI 1640 supplemented with 10% FBS at a concentration of 1x10^6 cells/ml, unless otherwise stated. Cells were transferred to 96 well microplates for cell proliferation assays, 24 well plates for caspase activity analysis and cell viability analysis, or to 6 well plates for two-dimensional electrophoresis. Cells were left to

equilibrate for 1 hr at 37°C before exposure to the isothiocyanates. Isothiocyanates were added directly to the cell culture medium.

2.3.2 Cell proliferation BrdU ELISA

A commercially available colorimetric BrdU enzyme linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Manheim, Germany) was used to measure cell proliferation. The assay is based on measuring the incorporation of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) into the DNA of proliferating cells (Hawker 2003). Plates (96 well) were seeded with 100 μL of a 1×10^6 cell/mL Jurkat culture and incubated at 37°C for 1 hr. The cells were then treated with a range of concentrations of the appropriate isothiocyanate (section 2.2.1) and incubated at 37°C for 30 min, unless otherwise stated. Each sample was then diluted to 5×10^5 cells/mL by taking 50 μL of each sample and transferring it to a new well containing 50 μL of fresh media. The diluted cells were immediately treated with 10 μL of the kit BrdU labelling reagent and incubated at 37°C for 30 min. The cells were then sedimented by centrifugation of the plate at 500 *g* for 10 min. Media was removed by inverting the plate. Cells were washed by addition of 100 μL PBS to each well followed by sedimentation by centrifugation of the plate at 500 *g* for 10 min. PBS was removed by inverting the plate and the washing process was repeated. As much PBS as possible was removed and the cells were dried using a hair-dryer for 10 min. Cells were incubated for 30 min at room temperature with 200 μL of the kit FixDenat solution to fix cells to the plate and expose the BrdU-labelled DNA. FixDenat was removed by inverting the plate and 100 μL of the kit anti-BrdU-peroxidase was added to each sample. The cells were incubated with the antibody solution for 90 min at room temperature. Excess antibody was removed by washing wells three times with 200 μL of the kit washing solution. The washing solution was removed by inverting the plate and gently tapping the plate on a paper-towel. Finally, 100 μL of the kit peroxidase substrate solution, containing tetramethyl benzadine, was added to each well and the rate of absorbance increase at 370 nm was measured. A blank, containing culture medium only, was carried out in each experiment to measure non-specific binding of BrdU and anti-BrdU-peroxidase to the wells. Furthermore, a background control, containing cells but no BrdU label, was also carried out in each experiment to

measure non-specific binding of the anti-BrdU-peroxidase to the cells in the absence of BrdU.

Proliferation was graphed as a percentage of the proliferation measured in cells that were not treated with an isothiocyanate. This was calculated according to the equation:

$$\text{Proliferation} = \frac{[\Delta A_{370}\,(\text{treated sample}) - \Delta A_{370}\,(\text{blank})] - \Delta A_{370}\,(\text{background control})}{[\Delta A_{370}\,(\text{untreated sample}) - \Delta A_{370}\,(\text{blank})] - \Delta A_{370}\,(\text{background control})} \times 100\%$$

IC_{50} values were generated using SigmaPlot (Version 8, Jandel Scientific). Data from three individual experiments were pooled and x and y values (for example Table 2.3) were fitted into a 4 parameter sigmoid curve equation:

$$y = y_0 + \frac{a}{1 + e^{-\left[\frac{x - x_0}{b}\right]}}$$

Concentration of phenethyl isothiocyanate (µM)	**Proliferation (% of control)**
0	100
2	80.5
5	26.2
10	22.9
15	20.6
20	5.0

Table 2.3: Example of data used to generate IC_{50} values

Corresponding values for a, b, x0 and y0 were generated (Figure 2.1)

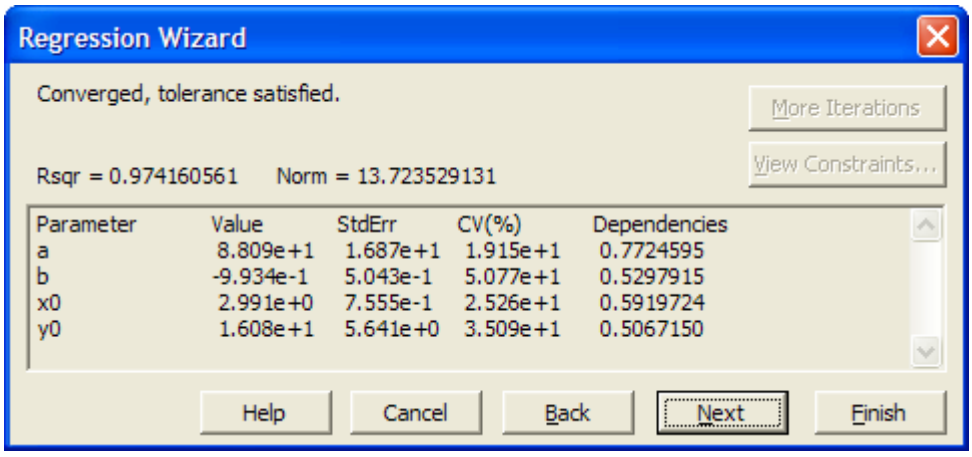


Figure 2.1: Example of the parameters for generation of IC_{50} values

To calculate the IC_{50} (x), y was set at 50 and values for a, b, x_0 and y_0 were substituted into the 4 parameter sigmoid curve equation. The standard error for the IC_{50} was calculated by multiplying the IC_{50} by the percentage variability about the parameter x_0 (CV%, Figure 2.1).

2.3.3 Cell viability assay

Propidium iodide (PI) is a dye that is excluded from viable cells but can penetrate the membranes of dead or dying cells. When inside a cell, PI binds to the major groove of DNA (Hudson, Upholt et al. 1969). This interaction facilitates the formation of a red fluorescent adduct. The proportion of cells that produce red fluorescence directly correlates to the proportion of cells that are non-viable following treatment with an isothiocyanate (Nicoletti, Migliorati et al. 1991). Wells of a 24 well plate were seeded with 1 mL of a 1×10^6 cell/mL culture and incubated at 37°C for 1 hr. The cells were then treated with a range of concentrations of the appropriate isothiocyanate (section 2.2.1) and incubated at 37°C for 24 hr. Cell samples were transferred to 5 mL plastic test tubes before 5 µL of a 1 mg/mL PI solution (section 2.2.2) was added directly to the culture medium. Samples were briefly vortexed, placed on ice and kept in the dark for 10 min. The proportion of PI-positive cells in a 10,000 cell sample was analysed using a FACSCalibre flow cytometer (Becton Dickinson, Mountain View, CA). Cells with PI fluorescence intensity over 20 units were counted as non-viable (Figure 2.2).

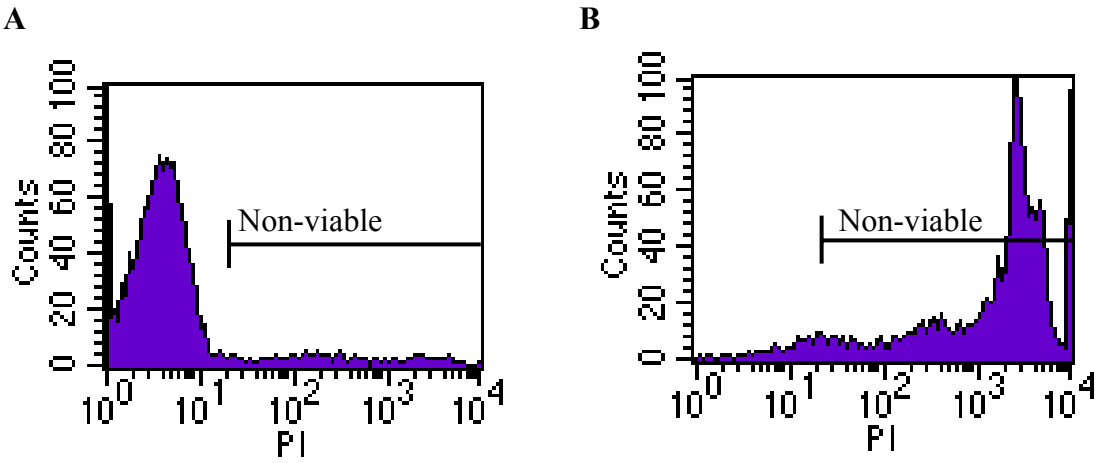


Figure 2.2: Representative graphs following PI analysis for loss of cell viability. (A) *Untreated sample and* ***(B)*** *sample treated with an isothiocyanate.*

Percentage loss of cell viability was adjusted in order that loss of cell viability in untreated samples was always equal to zero. Loss of cell viability was adjusted according to the equation below, where **(a)** represents the number of dead cells in treated samples and **(b)** represents the number of dead cells in the control sample:

$$\text{Percentage loss of cell viability} = (a\text{-}b/100\text{-}b) \times 100$$

LD_{50} values were generated using SigmaPlot (Version 8, Jandel Scientific), as outlined in section 2.3.2 for IC_{50} calculation.

2.3.4 Caspase activity assay

Caspase activation was monitored by measuring the fluorescence of amino-4-methylcoumarin (AMC) released following cleavage of the artificial caspase substrate Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) (Nicholson, Ali et al. 1995; Thornberry, Rano et al. 1997; Gorman, Hirt et al. 1999). Wells of a 24 well plate were seeded with 0.5 mL of a 1×10^6 cell/mL suspension and incubated at 37°C for 1 hr. Cells were treated with a range of concentrations of phenethyl isothiocyanate (section 2.2.1) and incubated for the desired time period at 37°C. Samples were transferred to 1.5 mL Eppendorf tubes and sedimented by centrifugation at 10,000 *g* for 15 sec. Culture medium was removed and the cell pellet stored at -80°C. Immediately prior to running the assay cell pellets were thawed and resuspended in 10 µL of PBS. The resuspended cells were transferred to a 96 well plate before addition of 90 µL of caspase buffer (section 2.2.3) containing 50 µM Ac-DEVD-AMC. The rate of release of fluorescent AMC was monitored for 30 min at 37°C using a POLARstar fluorescent platereader (BMG Labtechnologies Pty. Ltd. Mt Eliza, Australia) with an excitation wavelength of 390 nm and an emission/detection wavelength of 460 nm.

Caspase activity was expressed as a fold increase over the rate of caspase activity that could be detected in control cells. Immediately prior to treating cells with phenethyl isothiocyanate, a single control sample was removed in order to detect basal caspase activity in cultured cells. The fold increase in caspase activity was determined by dividing the rate of caspase activity in treated samples by the rate of caspase activity observed in control cells.

2.3.5 Induction of Fas-mediated apoptosis

The Fas-mediated extrinsic caspase activation pathway is induced by binding of Fas ligand to the corresponding Fas death receptor. Bcl-2 overexpressing cells are resistant to the induction of Fas-mediated apoptosis but can be sensitized by exposure to phenethyl isothiocyanate (Pullar, Thomson et al. 2004). The ability of other isothiocyanates to sensitize cells to Fas-mediated apoptosis was examined. Wells of a 24 well plate were seeded with 500 µL of a 5×10^6 cell/mL B9 culture and incubated at 37°C for 1 hr. Cells were treated with a range of concentrations of the appropriate isothiocyanate and incubated at 37°C for 1 hr. Isothiocyanate-treated cells were then either exposed to 4 µL of a 5 µg/mL activating Fas antibody solution or left without Fas antibody treatment. Cells were incubated for a further 4 hr at 37°C. Samples were transferred to 1.5 mL Eppendorf tubes and sedimented by centrifugation at 10,000 *g* for 15 sec. Culture medium was removed and the cell pellet stored at -80°C. Immediately prior to running the assay cell pellets were thawed and resuspended in 10 µL of PBS. The resuspended cells were transferred to a 96 well plate before addition of 90 µL of caspase buffer (section 2.2.3) containing 50 µM Ac-DEVD-AMC. The rate of release of fluorescent AMC was monitored for 30 min at 37°C using a POLARstar fluorescent platereader (BMG Labtechnologies Pty. Ltd. Mt Eliza, Australia) with an excitation wavelength of 390 nm and an emission/detection wavelength of 460 nm.

Caspase activity was expressed as a fold increase over caspase activity that could be detected in control cells. Immediately prior to treating cells with the isothiocyanates a single control sample was removed in order to detect basal caspase activity in cultured cells. The fold increase in caspase activity was determined by dividing caspase activity in treated samples by the caspase activity observed in control cells

2.3.6 Western Blotting for Bcl-2

The amount of Bcl-2 in Jurkat cells and Jurkat cells transfected with pCI-Neo/Bcl-2 cDNA was estimated by Western blotting (Burnette 1981), for Bcl-2. Two million cells were transferred to 1.5 mL Eppendorf tubes and sedimented by centrifugation at 10,000 *g* for 20 sec. Culture medium was removed before washing by addition of 1 mL PBS and centrifugation at 10,000 *g* for 20 sec. PBS was removed and cell pellets were resuspended in 60 µL of sample extract buffer (section 2.2.5). Samples were

incubated for 30 min at 4°C with occasional vortexing. Cellular debris was removed by centrifugation at 12,000 *g* for 3 min. A protein assay (section 2.3.7) of the resulting supernatant was carried out before samples were stored at –80°C until required.

The cell lysates were resolved by discontinuous SDS-PAGE (Laemmli 1970). Twenty μg of protein was combined with enough sample loading buffer (section 2.2.4) to bring the final volume to 35 μL. Samples were loaded onto a 4% stacking gel and resolved with a 15% separating gel (section 2.2.4). Electrophoresis was performed for 45 min at 200 V using the BioRad Mini Protean 3 system with a BioRad Powerpack 300 (Bio-Rad Laboatories, Hercules, CA, USA).

At the conclusion of SDS-PAGE the proteins were electotransferred for 50 min at 100 V to polyvinyldene difluoride (PVDF) membrane using a Bio-Rad Transblot apparatus (Bio-Rad Laboatories, Hercules, CA, USA). Non-specific antibody-binding sites were blocked by incubating the PVDF membranes with 5% milk powder (w/v) in Tris buffered saline with 0.1% Tween^{TM} 20 ($TBST_{20}$) (section 2.2.5) for 1 hr. The blots were incubated overnight at 4°C with a primary antibody against Bcl-2 diluted by a factor of 20,000 in $TBST_{20}$ containing 2% milk powder (w/v). The PVDF membranes were thoroughly washed with $TBST_{20}$ and then incubated for 1 hr with HRP-conjugated sheep anti-mouse antibody diluted by a factor of 20,000 in $TBST_{20}$ containing 2% milk powder (w/v). Secondary antibody was thoroughly washed off with $TBST_{20}$. Antibodies bound to the membrane were detected using an enhanced chemiluminescence system (ECL) (Amersham Biosciences), according to manufacturer's instructions and visualized using a Chemidoc FX imager (BioRad Laboratories, Hercules, CA, USA). Western blotting for β-actin was also carried out to ensure even protein loading between lanes. Following detection of Bcl-2 the ECL reagent was removed by thorough washing of the membranes in $TBST_{20}$. Non-specific antibody-binding sites were blocked by incubating the PVDF membranes with 5% milk powder (w/v) in $TBST_{20}$. The membranes were then incubated for 2 hr with a primary antibody against β-actin diluted by a factor of 5,000 in $TBST_{20}$ containing 2% milk powder (w/v). The membranes were thoroughly washed with $TBST_{20}$ and then incubated for 1 hr with HRP-conjugated sheep anti-mouse antibody diluted by a factor of 20,000 in $TBST_{20}$ containing 2% milk powder (w/v). Secondary

antibody was thoroughly washed off with $TBST_{20}$. Antibodies bound to the membrane were detected using an enhanced chemiluminescence system (ECL) (Amersham Biosciences), according to manufacturer's instructions and visualized using a Chemidoc FX imager (BioRad Laboratories, Hercules, CA, USA).

2.3.7 Protein assay

Sample protein concentration was estimated using a Bio-Rad Detergent Compatible (D_c) protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). A standard curve containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg bovine serum albumin (BSA) was transferred into a microplate along with 2 µL of sample lysates. Twenty-five µL of reagent S was added to each well, followed by 200 µl of reagent B. Wells were incubated at room temperature for 15 min before absorbance was read at 750 nm using a MRX microplate reader (Dynatech Laboratories). Sample protein concentration was calculated by comparison to the BSA standard curve.

2.3.8 Detection of oxidised thiol proteins by two-dimensional electrophoresis

Oxidised thiol proteins were monitored by using a proteomic technique developed by Dr James Baty (Baty, Hampton et al. 2002). Ten million B9 cells (1×10^6 cells/mL) were seeded into three wells of a six-well culture plate. Cells were left to incubate for 1 hr at 37°C. Cells were subsequently treated with either 15 µM phenethyl isothiocyanate or 15 µM sulforaphane (section 2.2.1) while 10 µL (0.1%) DMSO was added to control cells. Cells were incubated for 30 min at 37°C. Cells were harvested and pelleted by centrifugation at 1,000 *g* for 5 min. Culture medium was removed and the remaining pellet resuspended in 1 mL of ice-cold PBS. Suspended cells were centrifuged at 10,000 *g* for 15 sec. The PBS was aspirated and another 1 mL of ice-cold PBS was added to the resulting pellet. Once again cells were pelleted by centrifugation at 10,000 *g* for 15 sec and PBS was removed. The washed cell pellet was resuspended in 200 µL of NEM buffer (section 2.2.6) (final concentration 5×10^6 cells/mL) and kept on ice for 15 min. Cells were subsequently lysed by addition of 8 µL of a 25% CHAPS solution (final concentration of 1%). Cells were incubated with CHAPS for 15 min, with vortexing every 5 min. The lysed cell preparation was then centrifuged at 16,000 *g* for 4 min. Spin columns that had been pre-equilibrated with sample extract buffer (section 2.2.5) were loaded with 90 µL of the resulting

supernatant. Two spin columns were used for each sample. The spin columns were centrifuged at 1,000 *g* for 4 min and the resulting eluent was pooled. A protein assay (section 2.3.7) was carried out on the pooled samples. The extracts were then treated with 1 µL of a 1 M DTT preparation (final concentration of 5 mM) and kept on ice for 10 min. Four µL of a 10 mM IAF solution (section 2.2.6) (final concentration of 200 µM) was subsequently added and samples were incubated in the dark, at room temperature, for 10 min. The IAF-labelled samples were loaded onto spin columns that had been pre-equilibrated with sample extract buffer (section 2.2.5). Two spin columns were used for each sample. A maximum of 70 µL sample was loaded onto each column with the amount of protein kept constant between samples. The samples, containing a maximum of 500 µg protein, were made up to 300 µL with sample/rehydration solution (section 2.2.6) and delivered into the channels of a rehydration/equilibration tray. Immobilized pH gradient (IPG) strips (17 cm, pH 3-10) were placed directly over each sample. The IPG strips were overlayed with mineral oil to prevent evaporation. The sample was absorbed into the IPG strips overnight in the dark. Rehydrated strips were then transferred to the channels of a focussing tray. Pre-wetted electrode wicks were used to ensure good contact between the IPG strips and the electrodes of the focussing tray. The strips were overlaid with mineral oil and isoelectric focussing (IEF) was carried out on a Bio-Rad Protean IEF Cell (Bio-Rad Laboratories, Hercules, CA, USA) with the voltage increasing in a stepwise fashion (Table 2.4).

Step	Voltage (V)	Time (hr)
1	300	1
2	1000	1
3	3000	1
4	6000	12.5 (75,000 Vhr)
5	500	HOLD

Table 2.4: Conditions for isoelectric focussing.

The focussed IPG strips were transferred to a rehydration/equilibration tray and prepared for the second dimension by soaking for 15 min in equilibration buffer (section 2.2.6). The strips were transferred into clean channels of the tray and fresh

equilibration solution applied for another 15 min. For the second dimension, the strips were placed on 15% large-format SDS-PAGE gels (section 2.2.4). Strips were kept in place by overlaying with a 0.5% agarose solution (section 2.2.6). Electrophoresis was carried out for 16-18 hr in the dark with a constant current of 12 mA per gel using a Protean 2 Xi cell (Bio-Rad Laboratories, Hercules, CA, USA) with an Ephortec 3000 power supply. Gels were scanned using a Bio-Rad Molecular Imager® FX (Bio-Rad Laboratories, Hercules, CA, USA) with an excitation wavelength of 488 nm and an emission/detection wavelength of 530 nm.

2.3.9 Two-dimensional (2-D) gel image analysis

Gel images of resolved IAF-labelled thiol proteins were analysed with PDQuest™ version 7.1.1 2-D electrophoresis gel analysis software (Bio-Rad Laboratories, Hercules, CA, USA). Background fluorescence was filtered from the images using the software's default settings and the majority of spots were automatically detected. Manual editing of the detected spots was carried out to ensure that all spots were identified. Reference spots in a gel were matched with spots in the corresponding gels. The PDQuest™ analysis software was used to generate a list of spots with at least a two-fold increase or decrease in fluorescence in response to isothiocyanate exposure. Each spot that the software nominated was visually inspected to determine if the change was authentic or an artefact of spot detection. A consistent change was defined as a change that occurred in at least two out of three experiments. An estimation of spot molecular weights (MW) and isoelectric point (pI) was made by comparison with the positions of proteins with known MW and pI.

2.3.10 Silver stain procedure

Following visualization of IAF fluorescence, total protein content was visualized by silver staining. Gels were fixed with a 50% ethanol, 10% acetic acid solution for 30 min and then washed with a 5% ethanol, 1% acetic acid solution for 15 min. Gels were washed three times with milliQ water for 5 min each before addition of solution 1 (section 2.2.7) for 2 min. Gels were thoroughly washed three times with milliQ water for 30 sec and then incubated with solution 2 (section 2.2.7) for 30 min. Gels were washed twice with milliQ water for 20 sec each before addition of solution 3. Solution 3 (section 2.2.7) was quickly washed off with milliQ water as soon as colour development reached the desired intensity. Colour development was stopped by

addition of solution 4 (section 2.2.7). Gel images were acquired using a BioRad Fluor-S™ MultiImager (Bio-Rad Laboratories, Hercules, CA, USA).

Chapter 3

The ability of phenethyl isothiocyanate to induce apoptosis in cells overexpressing Bcl-2.

Results reported in this section are published in:
Thomson, S.J.*, **Brown, K.K.***, Pullar, J.M. and Hampton M.B. (2005) Phenethyl isothiocyanate triggers apoptosis in Jurkat cells made resistant by the overexpression of Bcl-2. *Cancer Research* **66**: 6772-6777 *Joint first authors

3.1 Introduction

The isothiocyanate family constitutes a large class of compounds that are abundant in a wide variety of cruciferous vegetables and possess potent cytotoxicity against cancer cells *in vitro* and *in vivo*. Phenethyl isothiocyanate (PEITC) (Figure 3.1) in particular has received much attention as a potential chemopreventive and chemotherapeutic agent (Chen et al. 2002; Solt et al. 2003; Kim et al. 2005; Yang et al. 2005; Khor et al. 2006).

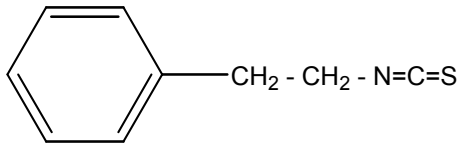


Figure 3.1: Chemical structure of PEITC *(adapted from Xu and Thornalley 2000a).*

PEITC is a major phytochemical constituent of watercress, generated upon enzymatic hydrolysis of the glucosinolate precursor gluconasturtiin. It is also present in many other cruciferous vegetables and is one of the most prominent isothiocyanates consumed in the human diet (Rose et al. 2005). PEITC is one of the most extensively studied isothiocyanates with respect to chemopreventive activity because of its high potency against a variety of tumours, and tumour cell lines, and also because of its low *in vivo* toxicity (Hecht 1995; NCI 1996; Rose et al. 2003; Zhang et al. 2003; Conaway et al. 2005).

Previous work from our laboratory has demonstrated that PEITC possesses an ability to sensitize Bcl-2 overexpressing cells to Fas-mediated apoptosis (Pullar et al. 2004). Subsequent experiments were undertaken to determine if PEITC could also sensitize the Bcl-2 overexpressing cells to chemotherapeutic drugs. However, these preliminary studies suggested that PEITC could directly trigger apoptosis in the resistant cells. The aim of this chapter was, therefore, to fully characterise the effect of PEITC on cell viability, induction of apoptosis and proliferation of cells over-expressing the anti-apoptotic protein Bcl-2.

3.2 Experimental approach

3.2.1 The experimental system – Bcl-2 overexpressing Jurkat T cells

A Jurkat T lymphoma cell line and Jurkat cells that had previously been stably transfected to express varying levels of the anti-apoptotic protein Bcl-2 were utilised (Bcl-2 overexpressing Jurkat clones were generated by Dr Susan Thomson). Briefly, the Bcl-2 coding region was subcloned from human Bcl-2 cDNA (pICbcl-2 obtained from Professor Suzanne Cory, Melbourne, Australia) into a pCIneo vector (Figure 3.2). The pCIneo vector carried the human cytomegalovirus (CMV) enhancer/promoter region to promote constitutive expression of the Bcl-2 DNA insert (Schmidt et al. 1990). A simian virus 40 (SV40) enhancer/promoter region regulated the expression of a neomycin phophotransferase gene. Expression of the neomycin gene in mammalian cells confers resistance to the antibiotic geneticin (G-418) (Davies and Jimenez 1980) and was therefore used as a marker for successful transfection. Jurkat cells were transfected with the pCIneo/bcl-2 construct and successfully transfected cells were selected using G-418.

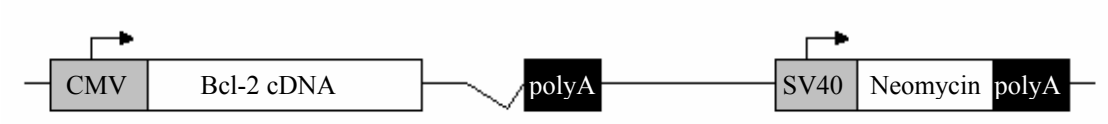


Figure 3.2: Schematic of the pCI-neo/bcl-2 cDNA construct used to generate Bcl-2 overexpressing Jurkat cells *(adapted from Brondyk 1994).* ***Abbreviations:*** *CMV=cytomegalovirus, polyA=polyadenylation signal, SV40= simian virus 40.*

3.2.2 Monitoring cell proliferation with a BrdU ELISA assay

Proliferation is the process by which cells divide to expand a population. Cellular proliferation requires the replication of DNA; therefore, DNA synthesis provides a parameter by which the rate of cell proliferation can be measured. DNA synthesis was quantified using a commercially available enzyme linked immunosorbent assay (ELISA) kit (section 2.3.2). The assay is based on the incorporation of the pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU) (Figure 3.3), in place of thymidine, into the DNA of proliferating cells, and the quantification of BrdU incorporation by ELISA (Hawker 2003).

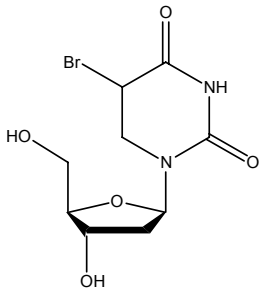


Figure 3.3: Chemical structure of BrdU *(adapted from Iball et al. 1966).*

Briefly, cells were incubated with PEITC before addition of BrdU. Actively replicating cells incorporated BrdU into newly synthesised DNA. DNA was then denatured to facilitate the binding of a peroxidase-conjugated anti-BrdU antibody. The amount of DNA-antibody complex was quantified by addition of the peroxidase substrate tetramethyl-benzidine (TMB). The developed colour directly correlated to the amount of DNA synthesis and hence to the number of proliferating cells in a population (Heil and Reifferscheid 1992; Maghni et al. 1999).

3.2.3 Measurement of loss of cell viability using propidium iodide

During cell death, the integrity of the cellular membrane is lost. While playing a crucial role in the breakdown of a cell, this process also allows the entry of otherwise membrane-impermeant compounds. Propidium iodide (PI) (Figure 3.4) is a polar dye that is excluded from viable cells but can penetrate the membranes of dead or dying cells (Wrobel et al. 1996).

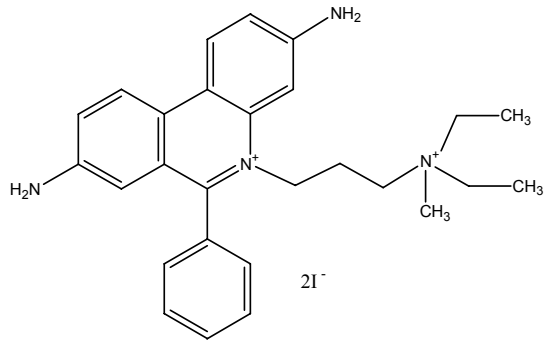


Figure 3.4: Chemical structure of PI *(adapted from Hudson et al. 1969).*

When inside a cell, PI binds to the major groove of DNA (Hudson et al. 1969). This interaction facilitates the formation of a red fluorescent adduct that can subsequently be visualised by flow cytometry (Nicoletti et al. 1991) (section 2.3.3). The proportion of cells that produce red fluorescence then directly correlates to the proportion of cells that are non-viable.

3.2.4 Measurement of caspase activity to determine induction of apoptosis

The caspases play a critical role in apoptosis. Caspase-mediated destruction of a cell is brought about by proteolytic cleavage of cellular targets C-terminal to specific aspartic acid residues. Caspase activity can be monitored by measuring the release of the fluorescent compound amino-4-methylcoumarin (AMC) from the artificial caspase substrate Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) (Nicholson et al. 1995; Gorman et al. 1999) (Figure 3.5).

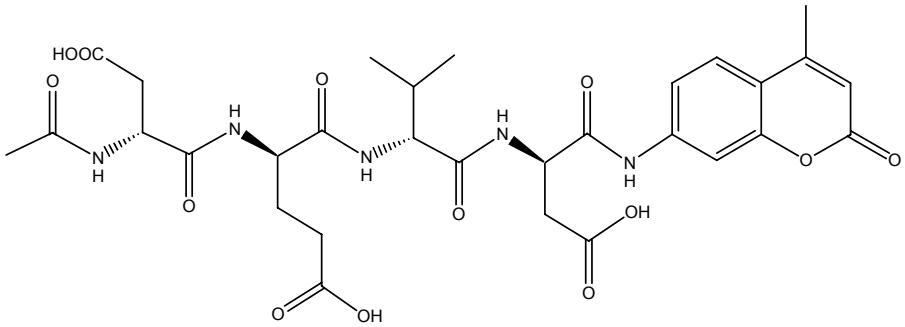


Figure 3.5: Chemical structure of the artificial caspase substrate Ac-DEVD-AMC *(adapted from Thornberry et al. 1997).*

Active caspases cleave the substrate to release Ac-DEVD and free AMC. The release of AMC can be measured by monitoring fluorescence intensity (section 2.3.4). The degree of fluorescence correlates to the amount of caspase activity.

3.3 Results

3.3.1 Inhibition of proliferation by PEITC

Initially, the proliferation of Jurkat cells in response to treatment with PEITC was examined. PEITC effectively inhibited the proliferation of Jurkat cells (Figure 3.6). After 1 hour of exposure to 2 μM PEITC, there was a small but significant inhibition of BrdU incorporation, increasing to 95% inhibition at 20 μM. The concentration of PEITC required to inhibit proliferation to 50% of control levels (IC_{50}) was 3.5 μM.

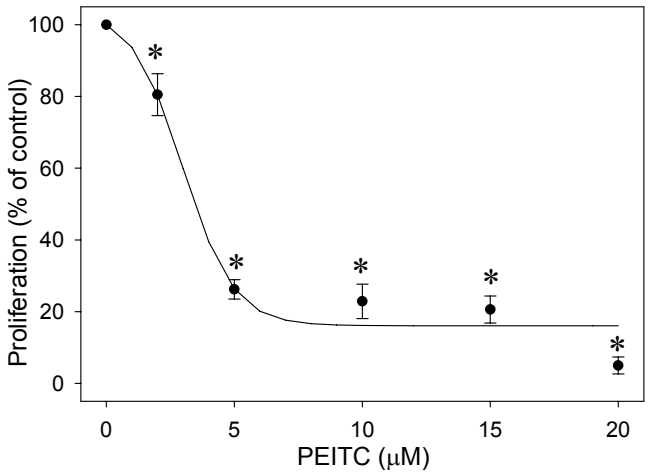


Figure 3.6: Inhibition of Jurkat cell proliferation in response to PEITC. *Cells were treated for one hour with PEITC. Cell proliferation was measured by monitoring the incorporation of BrdU into the DNA of proliferating cells using an ELISA (section 2.3.2). Plotted values represent mean ± standard error of three experiments. * Indicates a significant difference (P<0.05) from untreated cells (One Way Repeated Measures ANOVA with Bonferroni multiple comparison [SigmaStat, Jandel Scientific]).*

At later time points, the response of cells to concentrations above and below the IC_{50} was dramatically different (Figure 3.7). At 5 μM PEITC, the cells showed signs of recovery, with proliferation increasing two and three hours after treatment. However, this recovery did not occur at 15 μM PEITC, suggesting sustained growth arrest at higher concentrations.

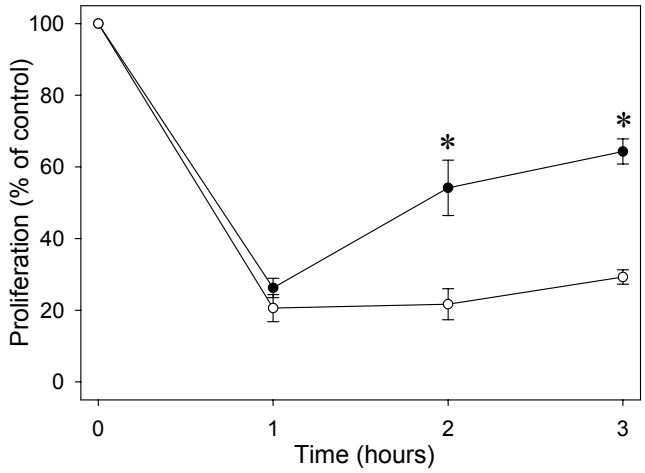


Figure 3.7: Inhibition of Jurkat cell proliferation in response to 5 µM or 15 µM PEITC. *Jurkat cells were treated for with 5 µM (●) or 15 µM (○) PEITC. Cell proliferation was measured by monitoring the incorporation of BrdU into the DNA of proliferating cells using an ELISA (section 2.3.2). Plotted values represent mean ± standard error of three experiments. * Indicates a significant difference (P<0.05) from cells treated for one hour (One Way Repeated Measures ANOVA with Bonferroni multiple comparison [SigmaStat, Jandel Scientific]).*

3.3.2 The effect of PEITC on Jurkat cell viability

A sustained decrease in cell proliferation can be associated with a loss in cell viability. For this reason, the viability of Jurkat cells following 24 hours treatment with PEITC was examined. Complete loss of Jurkat cell viability was induced upon exposure to 15 µM PEITC (Figure 3.8). The concentration of PEITC required to kill fifty percent of Jurkat cells (LD_{50}) was 7.4 µM.

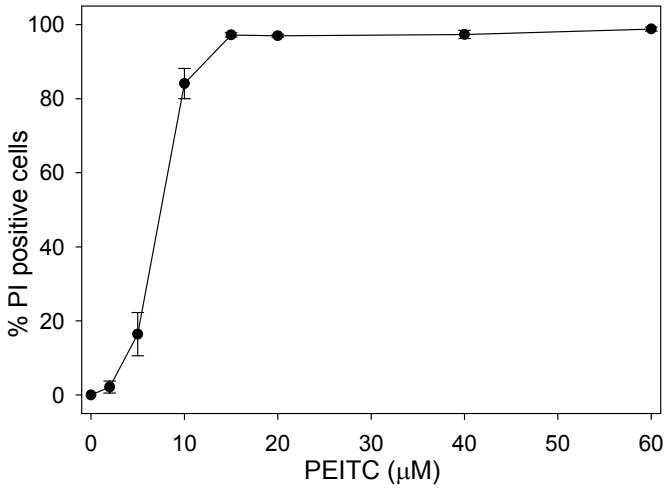


Figure 3.8: Viability of Jurkat cells in response to treatment with PEITC. *Jurkat cells were treated with varying concentrations of PEITC for 24 hours. Cell viability was then assessed by flow cytometric determination of the uptake of PI (section 2.3.3). Plotted values represent mean ± standard error of four experiments.*

3.3.3 Induction of apoptosis by phenethyl isothiocyanate

Although PEITC was shown to be completely cytotoxic towards Jurkat cells (section 3.3.2) the mechanism by which the cells were induced to undergo cell death was not identified. Controlled cell death by apoptosis is favourable; however, PEITC may induce Jurkat cell death by triggering an apoptosis-independent mechanism, such as necrosis (Majno 1995; Zeiss 2003). Normally, Jurkat cells present with a roughly spherical morphology (Figure 3.9). Following treatment with 10 µM PEITC, extensive cell shrinkage and budding of apoptotic bodies was evident (Figure 3.10), indicating induction of apoptosis in response to isothiocyanate exposure. Treatment of Jurkat cells with 40 µM PEITC resulted in cells with a swollen and granular appearance, which are characteristic morphological features of necrosis (Kerr 1971) (Figure 3.11).

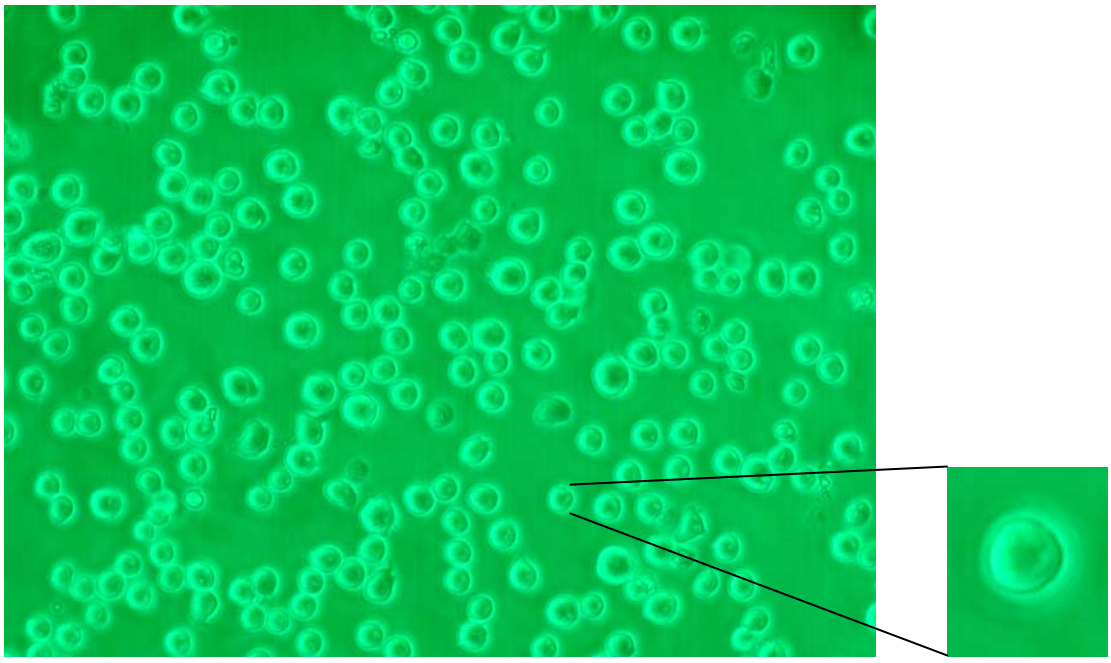


Figure 3.9: Jurkat cell morphology. *A zoom image of a normal Jurkat cell is shown. Image is representative of Jurkat morphology in three experiments. Image obtained using an inverted phase contrast microscope (Olympus Optical Ltd, Tokyo, Japan) at 200× magnification.*

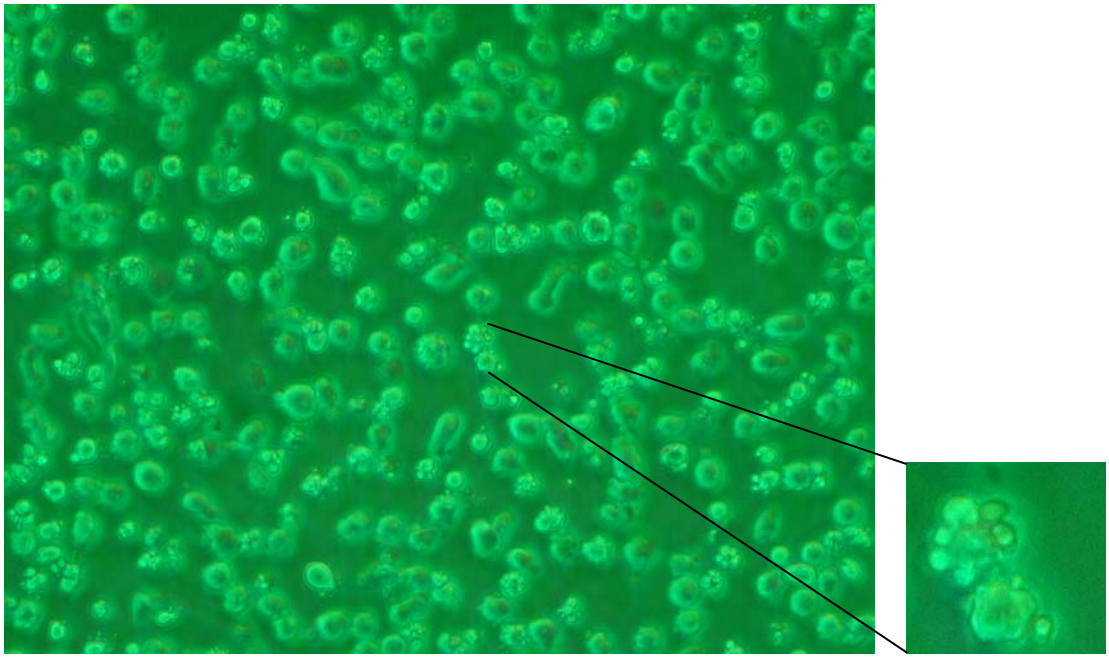


Figure 3.10: Apoptotic cell morphology following treatment with 10 µM PEITC. *Jurkat cells were treated with 10 µM PEITC for 3 hours. A zoom image of an apoptotic Jurkat cell is shown. Image is representative of Jurkat morphology in three experiments. Image obtained using an inverted phase contrast microscope (Olympus Optical Ltd, Tokyo, Japan) at 200× magnification.*

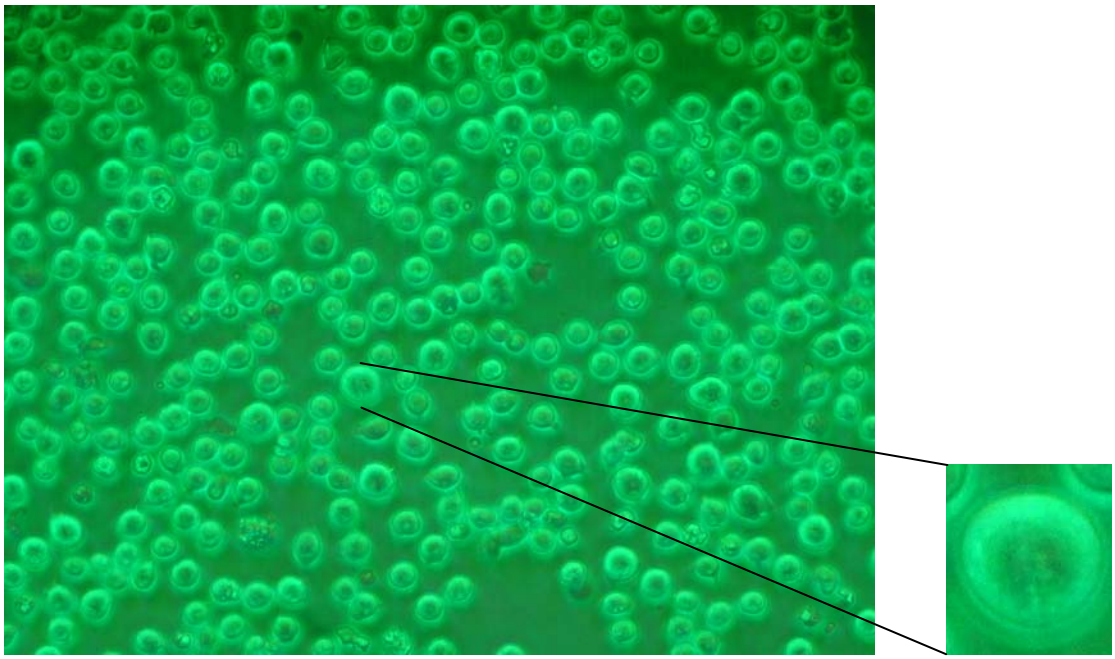


Figure 3.11: Necrotic cell morphology following treatment with 40 µM PEITC. *Jurkat cells were treated with 40 µM PEITC for 3 hours. A zoom image of a necrotic Jurkat cell is shown. Image is representative of Jurkat morphology in three experiments. Image obtained using an inverted phase contrast microscope (Olympus Optical Ltd, Tokyo, Japan) at 200× magnification.*

To confirm that PEITC could induce apoptosis in Jurkat cells, caspase activity was monitored. PEITC induced a time- and concentration-dependent caspase activation (Figure 3.12). Maximum caspase activity was measured with 10 μM PEITC and after four hours of treatment with PEITC. A sharp decrease in Jurkat caspase activity after 8 hours treatment with PEITC was evident, which is most likely due to a loss of cell viability.

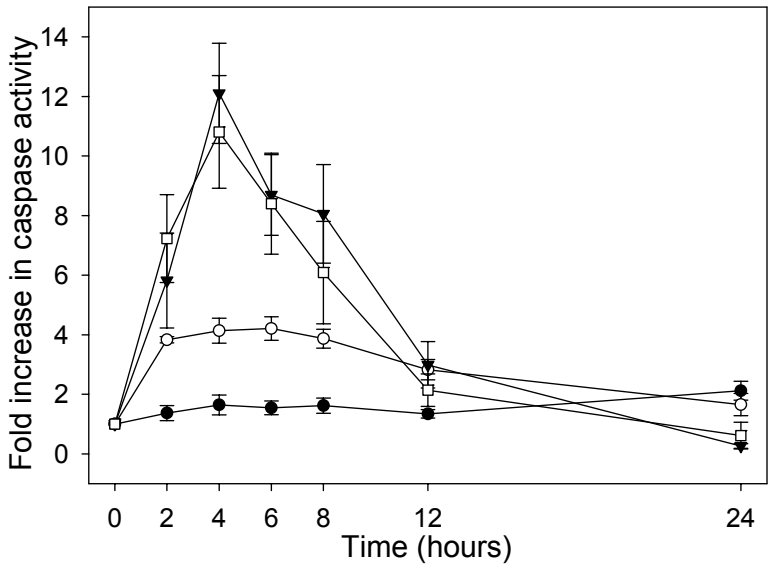


Figure 3.12: The time course of Jurkat caspase activity in response to PEITC. *Cells were treated with PEITC and samples were removed for caspase activity analysis 2, 4, 6, 8, 12, and 24 hours after treatment. Caspase activity was monitored by measuring the release of AMC from the artificial caspase substrate Ac-DEVD-AMC (section 2.3.4). Symbols represent: no PEITC (●), 5μM PEITC (○), 10 μM PEITC (▼) and 15μM PEITC (□). Plotted values represent mean ± standard error of at least four experiments. Caspase activity is expressed as a fold increase over the caspase activity that could be detected in control cells.*

It is interesting to note that PEITC concentrations found to be completely cytotoxic to the Jurkat cell line induce cell death via caspase activation and apoptosis (Figure 3.12). When the cells were treated with higher concentrations of PEITC (40 μM and 60 μM) caspase activity dropped back to control levels but maximum cell death was still induced. This result suggests that high concentrations of PEITC will cause a degree of damage that will induce cell death by necrosis, rather than via activation of the apoptotic pathway.

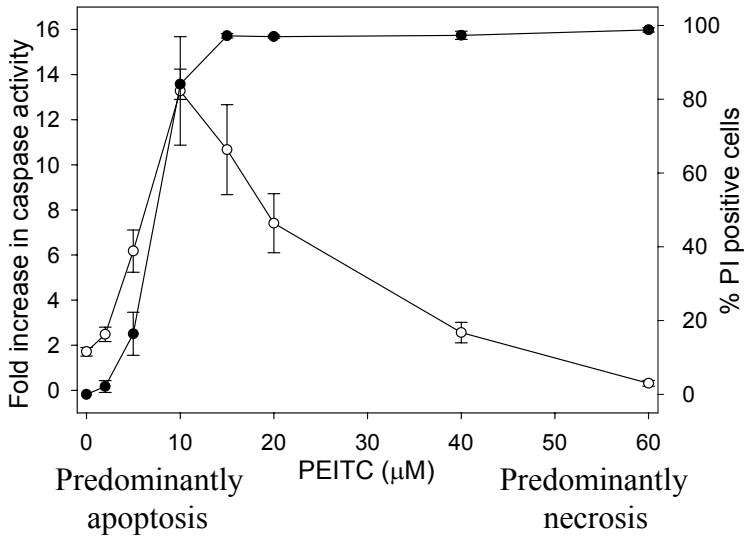


Figure 3.13: Loss of Jurkat cell viability is mediated via apoptosis at low concentrations of PEITC but by necrosis at high concentrations. *PEITC-treated Jurkat cells were removed for caspase activity analysis after 4 hours and for cell viability analysis following 24 hours of treatment. Cell viability (●) was assessed by flow cytometric determination of the uptake of PI (section 2.3.3) while caspase activity (○) was monitored by measuring the release of AMC from the artificial caspase substrate Ac-DEVD-AMC (section 2.3.4). Plotted values represent mean ± standard error of at least three experiments.*

3.3.4 The effect of PEITC on the viability of cells overexpressing Bcl-2

As discussed in Chapter One (section 1.4.1), overexpression of the anti-apoptotic protein Bcl-2 blocks apoptosis in response to numerous stimuli. The ability of PEITC to induce apoptosis in cells overexpressing Bcl-2 was examined using Jurkat clones that overexpressed varying levels of the anti-apoptotic protein Bcl-2 (section 3.2.1). Two control cell lines (the Jurkat cell line and an empty vector clone Neo2) and three Bcl-2 overexpressing cell lines (Bcl-2 #1, Bcl-2 #9 and Bcl-2 #38) were used. Western blotting for Bcl-2 protein demonstrated that the Bcl-2 #9 cell line (subsequently referred to as B9) expressed the highest amount of Bcl-2, with an approximately sixty-fold relative increase in Bcl-2 expression compared to the parental Jurkat cell line (Figure 3.14). The empty vector clone Neo1 was excluded due to relatively high apparent expression of Bcl-2 (Figure 3.14A).

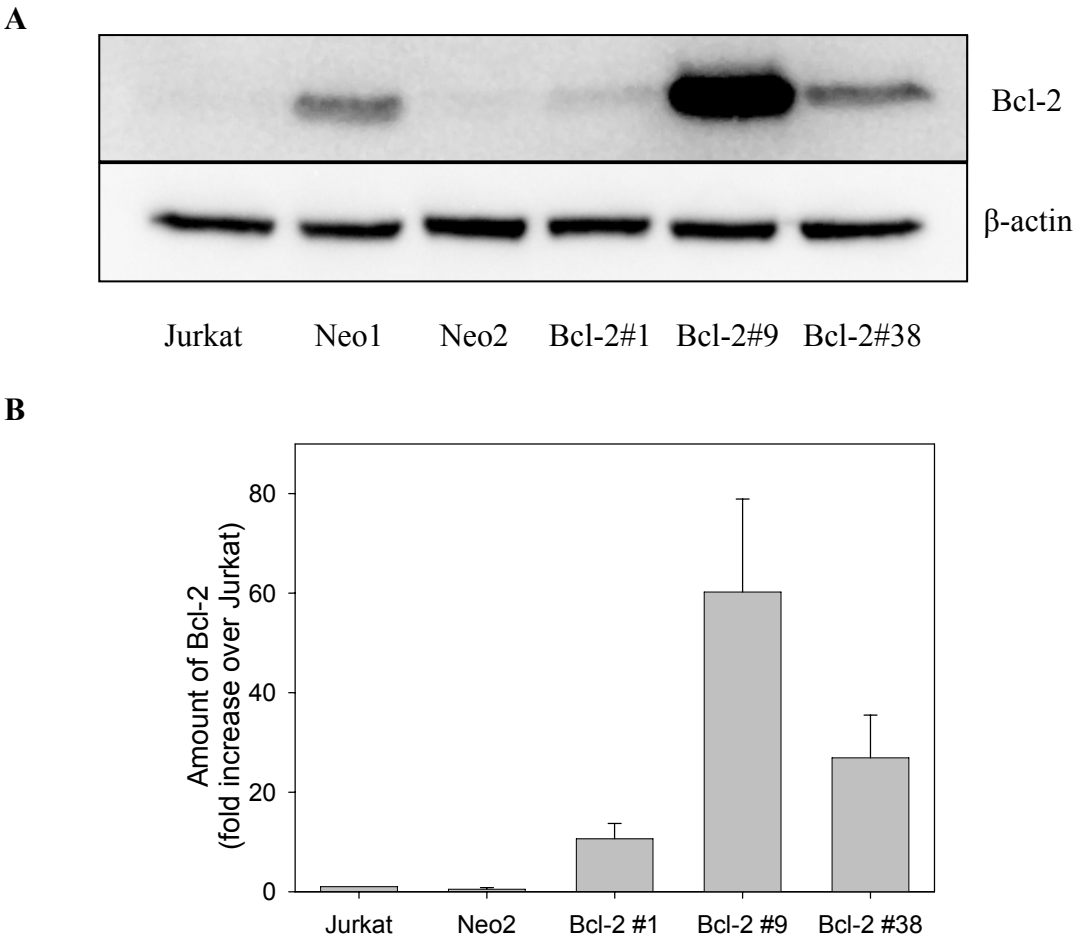


Figure 3.14: Bcl-2 overexpression in Jurkat T cells. *(A) Cell lysates from six clones were analysed by SDS-PAGE and immunoblotted for Bcl-2 and for β-actin as a loading control (section 2.3.6). Three experiments were performed and a representative blot is shown. (**B**) Quantification of the amount of Bcl-2 in the cell clones used during this study. The relative levels of Bcl-2 were assessed by densitometric analysis of Western Blots for Bcl-2 using Quantity One software. The amount of Bcl-2 is expressed as a fold increase over the amount of Bcl-2 detected in the Jurkat cell line. Plotted values represent the mean ± standard error of three experiments.*

The five cell lines were treated with PEITC for 24 hours before assessment of cell viability. The most significant outcome of the cell viability analysis was that all cell populations, regardless of the extent of Bcl-2 overexpression, could be induced to undergo complete loss of cell viability (Figure 3.15A). This is in contrast to previous experiments examining the effect of Bcl-2 expression on the efficacy of the standard chemotherapeutic drugs etoposide, cytosine arabinoside and melphalan (Thomson et al. 2005) (Figure 3.15B). Intermediate expression of Bcl-2 (Bcl-2#1 and Bcl-2#38) was shown to confer some resistance to the cytotoxicity of these chemotherapeutic

agents, whereas the degree of Bcl-2 expression in the B9 clone completely abrogated the cytotoxic potential of these agents.

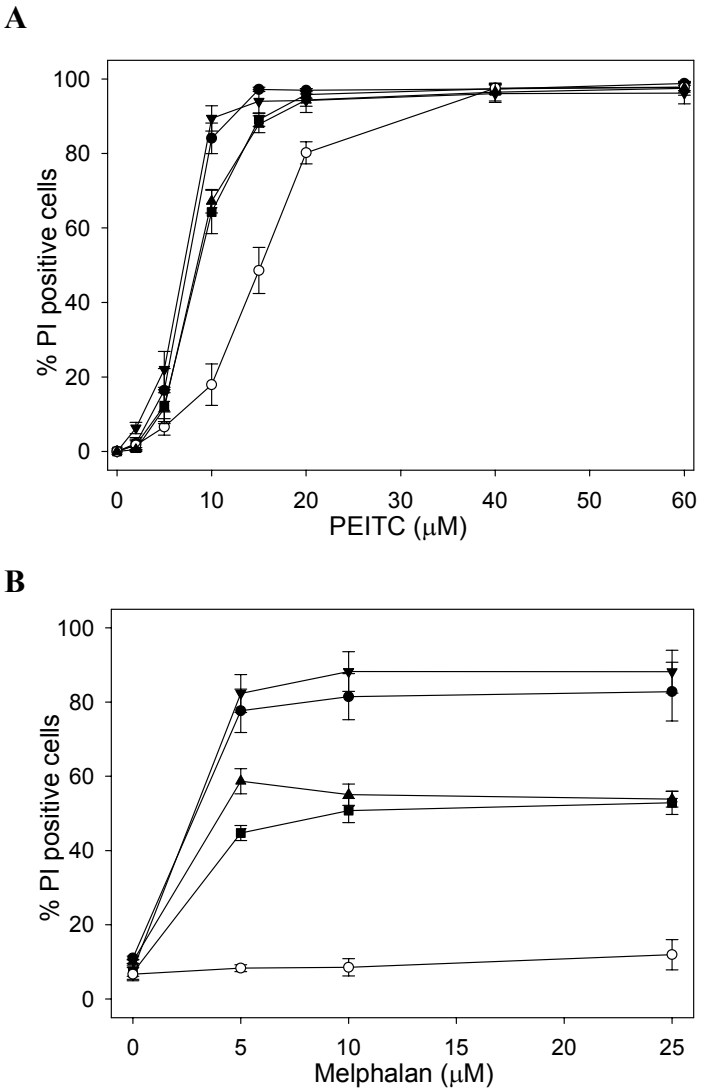


Figure 3.15: Cell viability of Jurkat clones expressing varying levels of Bcl-2 in response to treatment with PEITC and melphalan. ***(A)*** *All of the cell lines were treated with varying concentrations of (A) PEITC for 24 hours or (B) melphalan for 48 hours. Cell viability was then assessed by flow cytometric determination of the uptake of PI (section 2.3.3). Symbols represent Jurkat (●), Neo-2 (▼), Bcl-2 #1 (■), Bcl-2 #9 (○) and Bcl-2 #38 (▲). The experimental data for Figure B were generated by Dr Susan Thomson and Dr Juliet Pullar. Plotted values represent mean ± standard error of at least three experiments.*

The Jurkat clones that expressed intermediate levels of Bcl-2 (Bcl-2 #1 and Bcl-2 #38) demonstrated a slight resistance to PEITC, which is reflected by the raised LD_{50} values of 8.8 μM and 8.6 μM respectively, compared with an LD_{50} of 7.4 μM for the Jurkat cell line (Table 3.1). The cell line that expressed the highest amount of Bcl-2

had an LD_{50} value of 15.1 μM, approximately double the LD_{50} of the parental Jurkat cell line. A statistically significant correlation between the amount of Bcl-2 and the LD_{50} with PEITC was observed (Pearson correlation, P=0.01) (Figure 3.16); however, it would appear that only very large increases in the levels of Bcl-2 can confer any real degree of resistance against PEITC, given that the Bcl-2 #38 cells, which have approximately 27 times more Bcl-2 than the Jurkat control, have an LD_{50} that is raised by only 1.1 μM (Table 3.1).

Jurkat T cell clone	**Amount of Bcl-2**	**LD_{50} with PEITC (μM)**
Jurkat	1	7.4 ± 0.1
Neo 2	1 ± 0.5	6.7 ± 0.2
Bcl-2 #1	11 ± 3	8.8 ± 0.2
Bcl-2 #9	60 ± 19	15.1 ± 0.2
Bcl-2 #38	27 ± 9	8.6 ± 0.3

Table 3.1: The concentration of PEITC required to induce 50% loss of cell viability (LD_{50}) in Jurkat cells expressing varying levels of Bcl-2*. LD_{50} values were generated using Sigmaplot (Jandel Scientific). All values represent the mean ± standard error of at least three experiments.*

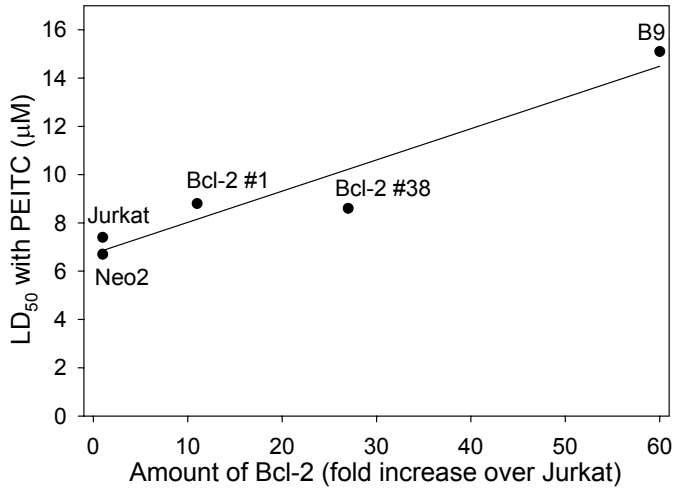


Figure 3.16: Correlation between the amount of Bcl-2 and the concentration of PEITC required to induce 50% loss of cell viability (LD_{50}). *Values from Table 3.1 are plotted. Pearson correlation, r^2=0.96, P=0.01 (SigmaStat, Jandel Scientific).*

3.3.5 The effect of Bcl-2 overexpression on induction of apoptosis by PEITC

Like the parental Jurkat cells, the Bcl-2 overexpressing cell lines were susceptible to the cytotoxic effects of PEITC. To confirm that PEITC was also capable of inducing apoptosis in cells that overexpress Bcl-2, caspase activation in the most resistant B9 cells was examined.

While the overexpression of Bcl-2 did reduce detectable caspase activity in response to PEITC, it could not prevent caspase activation (Figure 3.17). At all time points and concentrations of PEITC tested, the detectable caspase activity in the B9 cells did not reach the levels induced in the Jurkat cells (Figure 3.12 and Figure 3.17). Not only was the degree of caspase activity reduced, but the time of maximal caspase activation differed between the Jurkat and B9 cells. A maximum 6-fold increase in B9 caspase activity occurred between 8 and 12 hours after treatment with 15 μM PEITC. This is in contrast to a maximum 13-fold increase in Jurkat caspase activity after only 4 hours treatment with 10 μM PEITC.

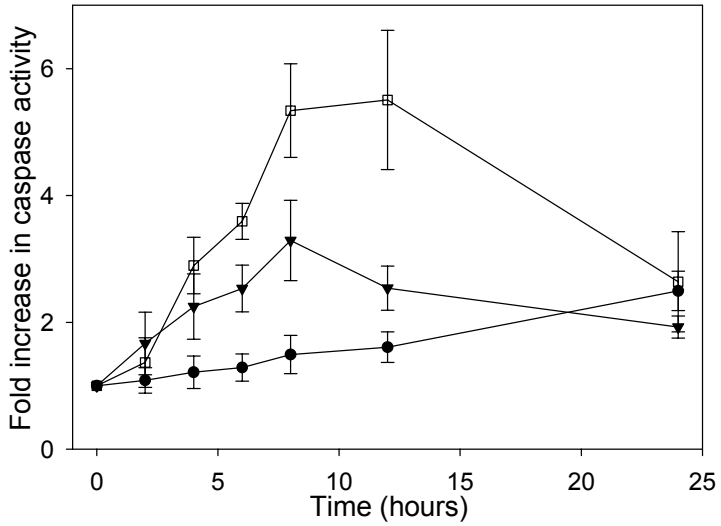


Figure 3.17: The time course of B9 caspase activity in response to PEITC. *Cells were treated with varying concentrations of PEITC and samples were removed for caspase activity analysis 2, 4, 6, 8, 12 and 24 hours after treatment. Caspase activity was monitored by measuring the release of AMC from the artificial caspase substrate Ac-DEVD-AMC (section 2.3.4). Symbols represent: no PEITC (●), 10 μM PEITC (▼) and 15μM PEITC (□). Plotted values represent mean ± standard error of at least four experiments. Caspase activity is expressed as a fold increase over the caspase activity that could be detected in control cells.*

Given that overexpression of Bcl-2 appeared to delay the onset of caspase activation, the concentration dependence of PEITC treatment on caspase activation at four hours, the time of peak caspase activity in Jurkat cells, and eight hours, the time of peak caspase activity in B9 cells, was examined. PEITC was able to induce caspase activation in all of the cell lines (Figure 3.18). In all cell lines, aside from the B9 cells, an approximately fifteen-fold increase in caspase activation was observed. The extent of Bcl-2 overexpression in the B9 cells decreased the maximum caspase activation to an approximately six-fold increase over control cells. Interestingly, the Bcl-2 #38 cell

line (Figure 3.18E), with a 27-fold increase in Bcl-2 expression, displayed a very similar profile of caspase activation to the Jurkat control cell line (Figure 3.18A). As was the case with cell viability (section 3.3.4), only a massive increase in Bcl-2 expression could alter the response of a cell towards PEITC (Figure 3.18D).

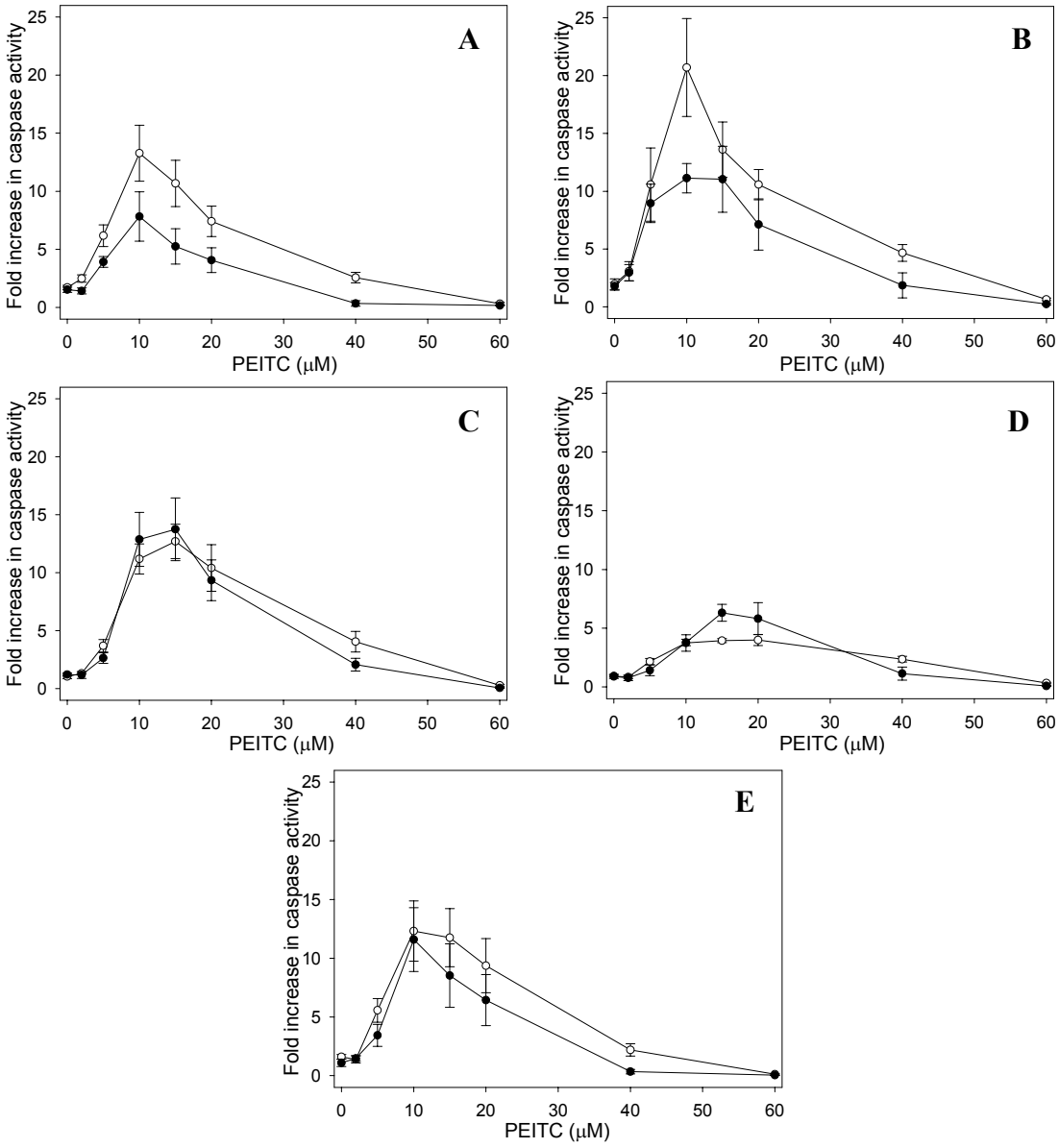


Figure 3.18: Caspase activity of Jurkat clones in response to four and eight hours of treatment with PEITC. *Cells were treated with increasing concentrations of PEITC and samples were removed after four (○) and eight (●) hours for caspase activity analysis. Caspase activity was monitored by measuring the release of AMC from the artificial caspase substrate Ac-DEVD-AMC (section 2.3.4). Graphed are* ***(A)*** *Jurkat,* ***(B)*** *Neo-2,* ***(C)*** *Bcl-2 #1,* ***(D)*** *B9 and* ***(E)*** *Bcl-2 #38. Plotted values represent mean ± standard error of at least four experiments. Caspase activity is expressed as a fold increase over the caspase activity that could be detected in control cells.*

Despite a reduction in the degree of caspase activation that could be induced in the B9 cells, the presence of caspase activity in response to PEITC is significant, given that previous studies demonstrated these cells to be completely resistant to the induction of apoptosis in response to traditional chemotherapeutic agents, including melphalan (Thomson et al. 2005) (Figure 3.19).

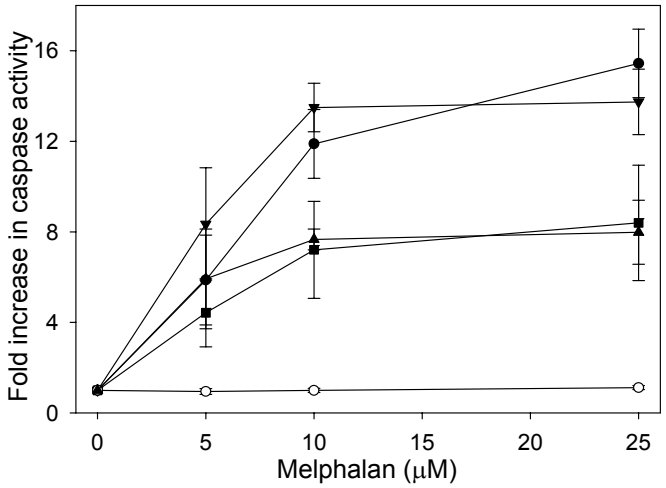


Figure 3.19: Induction of apoptosis by melphalan. *All cell lines were treated with increasing concentrations of melphalan. Samples were removed after 24 hours treatment for caspase activity analysis. Caspase activity was monitored by measuring the release of AMC from the artificial caspase substrate Ac-DEVD-AMC (section 2.3.4). Symbols represent Jurkat (●), Neo-2 (▼), Bcl-2 #1 (■), Bcl-2 #9 (○) and Bcl-2 #38 (▲). Plotted values represent mean ± standard error of three experiments. Caspase activity is expressed as a fold increase over the caspase activity that could be detected in control cells. Experimental data were generated by Dr Susan Thomson and Dr Juliet Pullar.*

As was the case with the Jurkat cells (Figure 3.13) the concentrations of PEITC found to be completely cytotoxic to the highly resistant B9 cells induced cell death via caspase activation and apoptosis (Figure 3.20). Once again, high concentrations of PEITC did not trigger caspase activation suggesting that cell death was a result of necrosis rather than through activation of the apoptotic pathway.

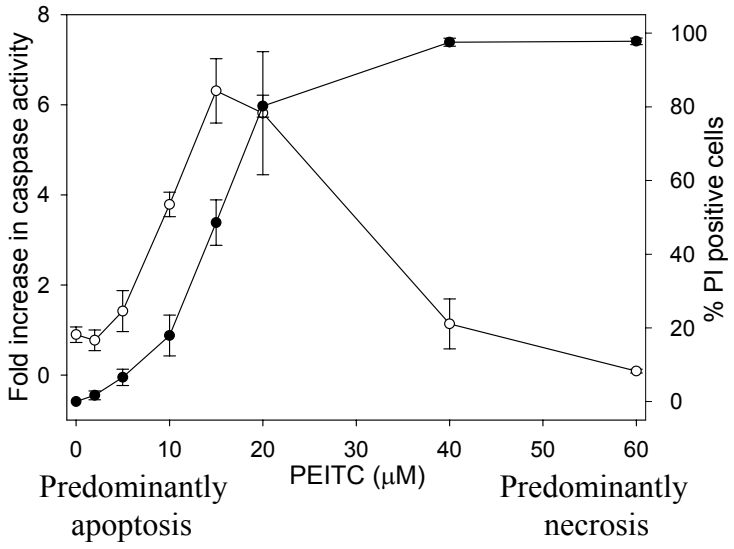


Figure 3.20: Loss of B9 cell viability is mediated via apoptosis at low concentrations of PEITC but by necrosis at high concentrations. *PEITC-treated B9 cells were removed for caspase activity analysis after eight hours and for cell viability analysis following twenty-four hours of treatment. Cell viability (●) was assessed by flow cytometric determination of the uptake of PI (section 2.1.3) while caspase activity (○) was monitored by measuring the release of AMC from the artificial caspase substrate Ac-DEVD-AMC (section 2.3.4). Plotted values represent mean ± standard error of at least three experiments.*

3.4 Discussion

In this section of the study, the ability of PEITC to inhibit cell proliferation, trigger caspase activation and kill target cells was investigated. PEITC was initially observed to rapidly inhibit proliferation of Jurkat cells. Very low concentrations of PEITC inhibited cell proliferation within one hour of exposure, suggesting that inhibition of proliferation is an early event mediated by the isothiocyanate. At concentrations less than 5 μM, this was a reversible effect, and cells continued proliferating at later times. Therefore, low doses of PEITC may trigger transient growth arrest but do not appear to induce progression to cell death. At doses of 5 μM to 20 μM, the inhibition of proliferation appeared to be a permanent effect and was closely associated with subsequent induction of apoptosis and loss of cell viability. At doses in excess of 20 μM, the loss in cell viability was predominantly caspase independent, and associated with necrosis.

Few studies have directly examined the ability of the isothiocyanates to inhibit cell proliferation by monitoring inhibition of DNA synthesis. PEITC has been shown to dramatically reduce DNA synthesis in human acute promyelocytic leukemia HL-60

and human myeloblastic leukemia ML-1 cells with IC_{50} values of 2.24 μM and 2.42 μM respectively (Xu and Thornalley 2000b). Inhibition of DNA synthesis has also been demonstrated in human colon adenocarcinoma CaCo2 cells with an IC_{50} of 2.4 μM (Visanji et al. 2004). An *in vivo* study has also shown that the *N*-acetyl cysteine conjugate of PEITC (PEITC-NAC) can reduce the number of actively proliferating cells in tumour tissue samples by as much as 75% (Chiao et al. 2004). In the cell lines described above, the inhibition of DNA synthesis was examined at much later time points than investigated in this study. We have demonstrated, for the first time, that low doses of PEITC can significantly reduce cell proliferation within one hour of treatment.

The majority of studies relating to the anti-proliferative efficacy of the isothiocyanates have focussed on monitoring inhibition of cell cycle progression. Generally, isothiocyanate administration results in G_2/M cell cycle arrest, which is the point in the cell cycle that is most often bypassed by cancerous cells (Malumbres and Barbacid 2001; Jackson and Singletary 2004; Singh et al. 2004; Srivastava and Singh 2004; Tang and Zhang 2004; Visanji et al. 2004). Such an arrest prevents mitosis and, therefore, the successful division of daughter cells. G_2/M arrest by the isothiocyanates has also been demonstrated *in vivo* (Srivastava et al. 2003). Interestingly, G_2/M phase arrested cells have been shown to be more sensitive to undergoing apoptosis in response to continued exposure to isothiocyanates (Miyoshi et al. 2004b). The exact mechanism by which the isothiocyanates can block cell cycle progression remains elusive; however, the expression and activity of numerous proteins that play a key role in the progression of the cell cycle have been shown to be modulated upon treatment with various isothiocyanates (Powolny et al. 2003; Xiao et al. 2003; Fimognari et al. 2004b).

While capable of inhibiting the proliferation of Jurkat cells, a dramatic loss of cell viability via the induction of apoptosis was also demonstrated following exposure to PEITC. A collection of studies suggest that induction of cell death by PEITC is not cell line specific (Table 3.2).

Cell type	Description	LD_{50} PEITC (μM)	Reference
Jurkat	T-cell leukemia	7.4	This study
		25	Chen, et al. 1998
HL-60	Acute promyelocytic leukemia	2.9	Adesida, et al. 1996
		5	Xu & Thornalley 2000b
		3.6	Zhang, et al. 2003
		1	Jakubikova, et al. 2005
HL-60/AR	Doxorubicin-resistant leukemia	4.9	Zhang, et al. 2003
HL-60/ADR	Multidrug-resistant leukemia	3.8	Jakubikova, et al. 2005
HL-60/VCR		2.1	Jakubikova, et al. 2005
8226	Myeloma	3.1	Zhang, et al. 2003
8226/Dox40	Doxorubicin-resistant myeloma	4.6	Zhang, et al. 2003
ML-1	Myeloblastic leukemia	3.3	Xu & Thornalley 2000b
PC-3	Prostate cancer	10	Xu, et al. 2005b
DU145		7	Xiao, et al. 2005a
LNCaP		7	Xiao, et al. 2005a
UM-UC-3	Bladder carcinoma	11.6	Tang & Zhang 2004
HepG2	Hepatoma	20	Rose, et al. 2005
		15	Rose et al. 2003
		11.2	Zhang, et al. 2003
PLC/PRF/5		4	Wu, et al. 2005
HT-29	Colorectal cancer	25	Hu, et al. 2003
		9.6	Zhang, et al. 2003
CaCo2	Colon adenocarcinoma	12	Bonnesen, et al. 2001
LS-174		20	Bonnesen, et al. 2001
HeLa	Cervical squamous carcinoma	7.5	Yu, et al. 1998
MCF-7	Breast cancer	11	Zhang, et al. 2003
HaCaT	Transformed epidermal keratinocytes	6.1	Zhang, et al. 2003

Table 3.2: Summary of previous studies examining cell death in response to treatment with PEITC.

In contrast to results from this investigation, an LD_{50} of 25 μM was previously measured in Jurkat cells treated with PEITC (Chen et al. 1998). However, in this study, cell viability was crudely measured by a trypan blue exclusion assay, which is far less sensitive to loss of cell viability than monitoring incorporation of PI by flow cytometry (Altman et al. 1993). Interestingly, PEITC is equally effective in cell lines derived from either solid or haematological malignancies, highlighting its potential application to the treatment of a wide variety of cancers.

In this study, PEITC also demonstrated a powerful ability to induce cell death despite extensive overexpression of the anti-apoptotic protein Bcl-2. Importantly, the loss of cell viability that was triggered by PEITC could be attributed to induction of apoptosis. The finding that PEITC could induce apoptosis and death of cells overexpressing Bcl-2 is in agreement with a recent study which showed that the isothiocyanate sulforaphane can directly induce apoptotic cell death in human prostate

cancer PC-3 cells that overexpress Bcl-2 (Singh et al. 2005). On the other hand, the results disagree with a study by Chen and colleagues showing that overexpression of Bcl-2 could prevent the induction of apoptosis by PEITC (Chen et al. 1998). As the study was carried out in embryonic kidney 293 cells, this disparity may reflect a difference in cell types. However, this study examined a single concentration of 20 μM PEITC. At the chosen concentration, control cells had lost 40% viability but there was no killing observed in the Bcl-2 overexpressing cells. Results from this study suggest that overexpression of Bcl-2 can shift the dose-response curve rather than confer protection against PEITC. For example, it was shown that 10 μM PEITC can kill 40% of Jurkat cells but can induce loss of cell viability in less than 20% of B9 cells. If this was the only concentration of PEITC examined the incomplete conclusion would be that Bcl-2 protects against induction of cell death by PEITC.

The results of this section of the study suggest that isothiocyanates may be useful for targeting cells that overexpress Bcl-2. To provide further insight into the mechanism of action of PEITC, an investigation of the cytotoxic activity of a selection of isothiocyanates was undertaken and is reported in Chapter Four.

Chapter 4

Structure-activity relationships of the isothiocyanates

Results reported in this section are published in:
Thomson, S.J.*, **Brown, K.K.***, Pullar, J.M. and Hampton M.B. (2005) Phenethyl isothiocyanate triggers apoptosis in Jurkat cells made resistant by the overexpression of Bcl-2. *Cancer Research* **66**: 6772-6777 *Joint first authors

4.1 Introduction

More than 120 naturally occurring isothiocyanates have been identified (Fahey et al. 2001). As discussed in Chapter One (section 1.1.2) there is considerable structural diversity in the side chains that can be associated with the isothiocyanate group. This diversity arises during the biosynthesis of glucosinolate precursors. Glucosinolate biosynthesis involves four key processes: 1) chain elongation of the amino acids alanine, leucine, isoleucine, valine, phenylalanine, tyrosine or tryptophan; 2) oxidative decarboxylation of these amino acids to generate an aldoxime; 3) conversion of the oxime into the basic glucosinolate structure; and (4) secondary modification of the glucosinolate by processes including oxidation, hydroxylation, sulfation and glucosylation (Halkier and Du 1997; Chen and Andreasson 2001). The processes of chain elongation and secondary modification allow for a vast variety of structures and a multitude of potentially biologically active compounds. Furthermore, a number of synthetic isothiocyanates have been produced in an attempt to improve chemopreventive activity (Morse et al. 1991; Fuke et al. 1997).

The electrophilic carbon residue of the isothiocyanate moiety is primarily responsible for mediating the biological effects of the isothiocyanates. However, the side chain attached to the functional group will also play a role in biological activity. The isothiocyanate side chain will influence electrophilicity of the –N=C=S group, steric hindrance, and lipophilicity (Zhang et al. 2005).

Many studies have already highlighted the different potencies of structurally diverse isothiocyanates both *in vitro* and *in vivo* (Hecht 2000; Conaway et al. 2002). The purpose of this section of the thesis was to carry out a structure-activity investigation

of a selection of isothiocyanates to determine key structural features that confer apoptosis-inducing properties in cells overexpressing Bcl-2.

4.2 Results

4.2.1 Requirement of the isothiocyanate moiety for cytotoxic activity

The cytotoxicity of PEITC was demonstrated in Chapter Three. To confirm that the isothiocyanate moiety is required for cytotoxic activity, benzyl isothiocyanate, benzyl thiocyanate and benzyl selenocyanate were compared (Figure 4.1). Although benzyl isothiocyanate (Ph-CH_2-N=C=S) and benzyl thiocyanate (Ph-CH_2-S=C=N) have the same chemical formula, they differ in the orientation of the –NCS atoms, an alteration that has a significant effect on the reactivity of the central carbon atom. The carbon between the nitrogen and the sulfur in benzyl isothiocyanate is more electrophilic than the corresponding carbon in benzyl thiocyanate (personal communication with Prof. Margaret Brimble, Department of Chemistry, University of Auckland). Benzyl selenocyanate (Ph-CH_2-Se=C=N) differs from benzyl isothiocyanate in that the sulfur atom of benzyl isothiocyanate is replaced by a selenium atom and the functional group is in the thiocyanate conformation. Although both selenium and sulfur are nucleophilic, this alteration abolishes the isothiocyanate functional group and changes the chemistry of the thiocyanate moiety (Jacob and Sies 2003).

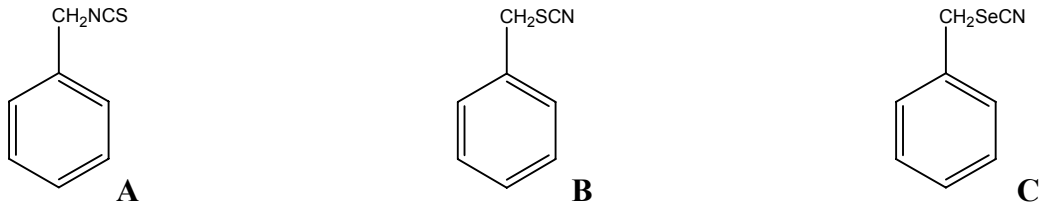


Figure 4.1: Structures of (A) benzyl isothiocyanate, (B) benzyl thiocyanate, and (C) benzyl selenocyanate.

Similar to PEITC (section 3.3.4), benzyl isothiocyanate was able to induce complete loss of cell viability in both the parental Jurkat cells and in the Bcl-2 overexpressing B9 cells, although it was less effective against the B9 cells than PEITC (Figure 4.2A). However, benzyl thiocyanate was only partially toxic towards the Jurkat cells and completely ineffective against the B9 cells (Figure 4.2B). Although benzyl thiocyanate demonstrated some cytotoxic activity towards the Jurkat cells, the profile of cell death was unusual in that maximum loss of cell viability (30% non-viable) was

observed when cells were treated with 10 μM benzyl thiocyanate and did not differ with concentrations up to 60 μM. This suggests that the mechanism of cytotoxicity of benzyl thiocyanate is different from that of benzyl isothiocyanate. Interestingly, benzyl selenocyanate displayed a relatively similar cell death profile to benzyl isothiocyanate in both the Jurkat and B9 cells (Figure 4.2C). The fact that benzyl thiocyanate has limited cytotoxicity while benzyl selenocyanate is highly cytotoxic suggests that cell death may be a result of the inherent cytotoxicity of seleno-compounds (Weiller et al. 2004).

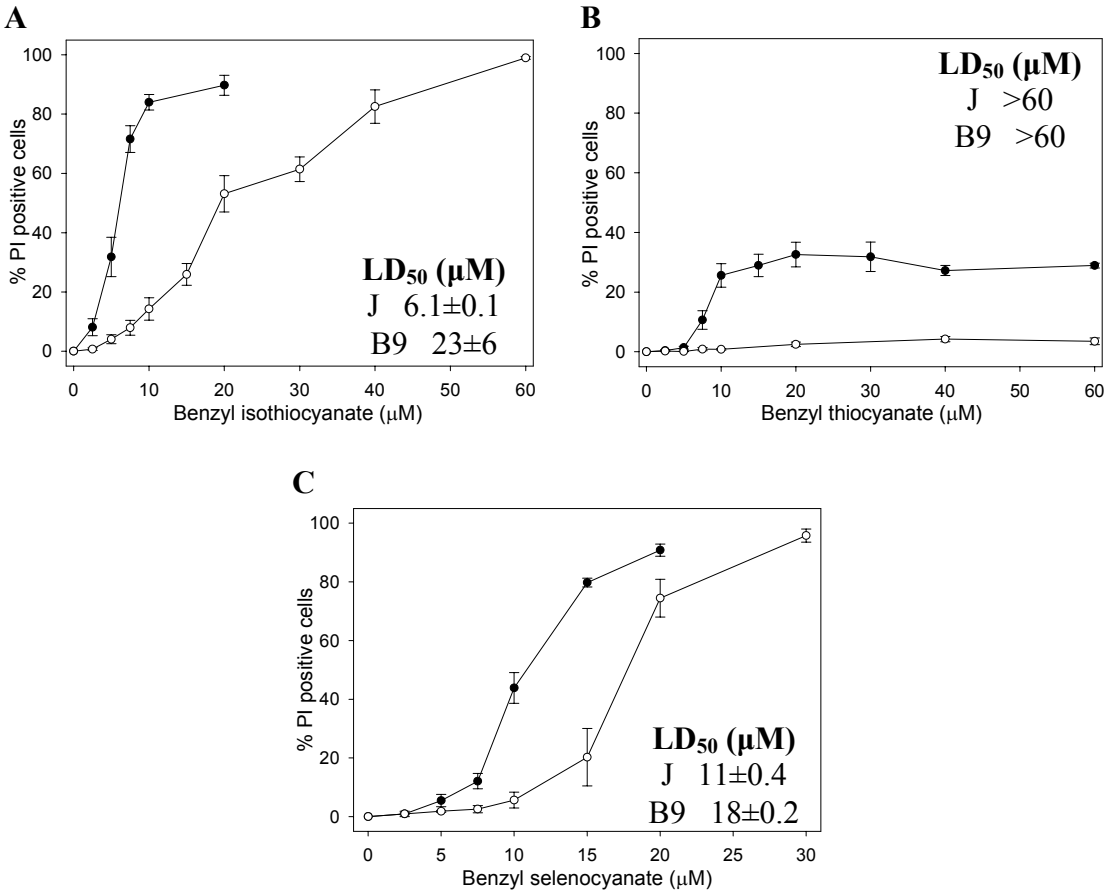


Figure 4.2: The cytotoxicity of benzyl isothiocyanate, benzyl thiocyanate and benzyl selenocyanate*. Both Jurkat (●) and B9 (○) cells were treated for 24 hours with either benzyl isothiocyanate* ***(A)****, benzyl thiocyanate* ***(B)*** *or benzyl selenocyanate* ***(C)*** *before analysis of cell viability by flow cytometric determination of the uptake of PI (section 2.3.3). Plotted values represent the mean ± standard error values of at least three experiments. LD_{50} values were generated using Sigmaplot Version 8.*

4.2.2 Structure-activity relationship of aromatic isothiocyanates

The cytotoxicities of a selection of aromatic isothiocyanates (Figure 4.3) were examined in order to determine the length of the carbon linker group, between the

phenyl ring and the isothiocyanate moiety that confers the most potent cytotoxic activity. *R*(-)-α-Methylbenzyl isothiocyanate (Figure 4.3G), which is closely related to PEITC, was also chosen to determine how the orientation of the isothiocyanate moiety with respect to the aromatic ring can affect cytotoxic activity.

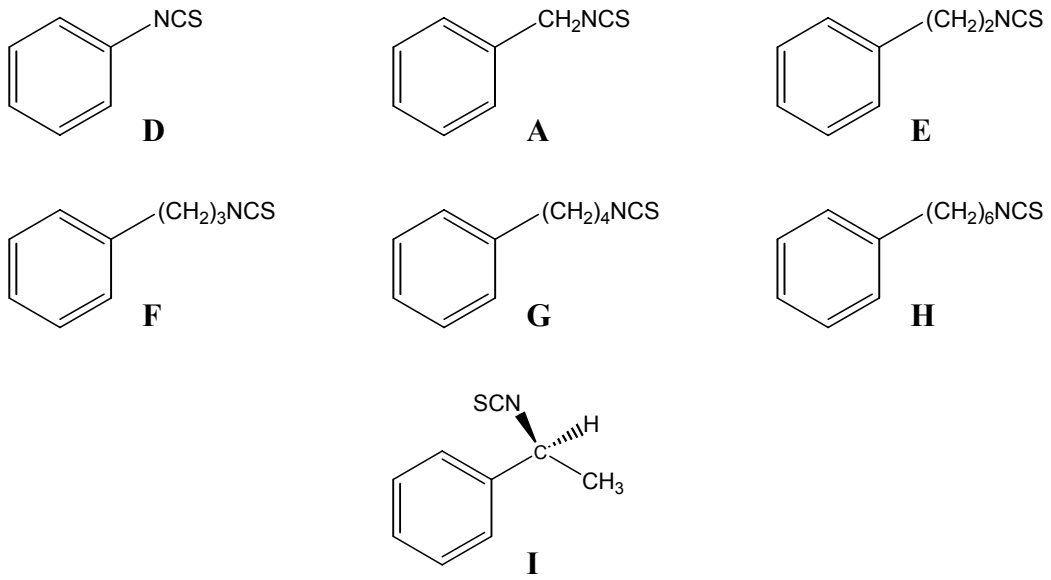


Figure 4.3: Structures of aromatic isothiocyanates. (D) *Phenyl isothiocyanate,* ***(A)*** *benzyl isothiocyanate,* ***(E)*** *PEITC,* ***(F)*** *3-phenylpropyl isothiocyanate,* ***(G)*** *4-phenylbutyl isothiocyanate,* ***(H)*** *phenylhexyl isothiocyanate and* ***(I)*** R*(-)-α-methylbenzyl isothiocyanate.*

Strikingly, phenyl isothiocyanate was incapable of inducing cell death in either the B9 cells or the sensitive Jurkat cells at concentrations up to 60 μM (Figure 4.4A). As highlighted earlier, benzyl isothiocyanate and PEITC (Figure 4.4B and 4.4C) displayed a very similar cell death profile in both Jurkat and B9 cells although, while benzyl isothiocyanate was more cytotoxic to Jurkat cells, PEITC was better at killing the B9 cells. Increasing the length of the carbon linker group beyond two CH_2 units adversely affected the ability of the isothiocyanates to induce cell death, although to a minimal extent considering the resistance of the B9 cells (Figure 4.4D-4.4F). Finally, altering the conformation of PEITC to give *R*(-)-α-methylbenzyl isothiocyanate significantly decreased cytotoxic activity in the B9 cells but did not dramatically reduce activity in the Jurkat cells (Figure 4.4G). These results emphasise the requirement for quite rigid structural features when targeting cells overexpressing Bcl-2.

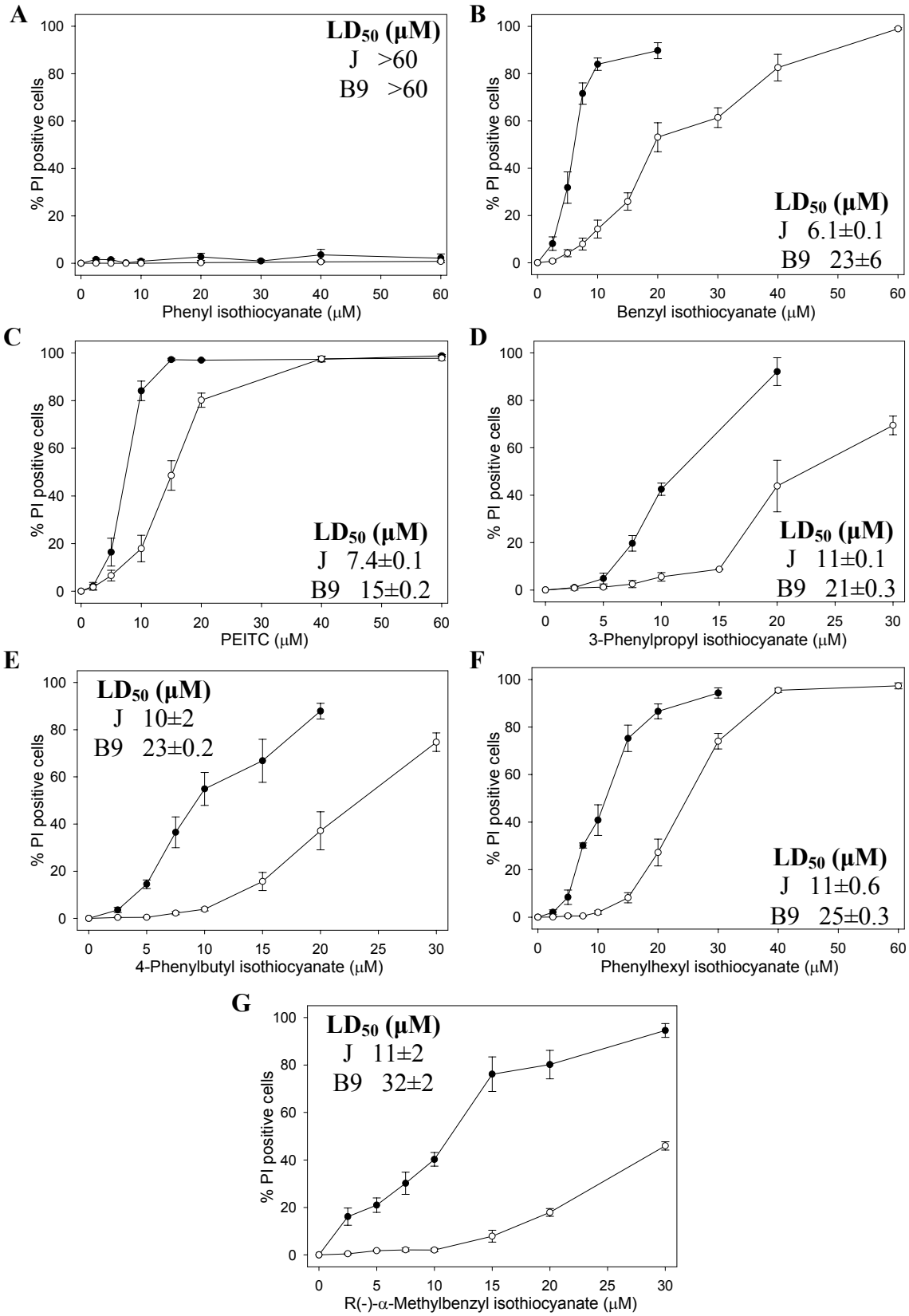


Figure 4.4: The cytotoxicity of aromatic isothiocyanates*. Jurkat (●) and B9 (○) cells were treated for 24 hours with phenyl **(A)**, benzyl **(B)**, phenethyl **(C)**, 3-phenylpropyl **(D)**, 4-phenylbutyl **(E)**, phenylhexyl **(F)** or R(-)-α-methylbenzyl **(G)** isothiocyanate before analysis of cell viability by flow cytometric determination of the uptake of PI (section 2.3.3). Plotted values represent the mean ± standard error of at least three experiments. LD_{50} values were generated using Sigmaplot Version 8.*

4.2.3 Cytotoxicity of the products of aromatic isothiocyanate metabolism

Upon entering a cell, an isothiocyanate will rapidly react with intracellular GSH giving rise to a dithiocarbamate (Zhang et al. 1995). This reaction initiates the cellular efflux and metabolism of the isothiocyanate via the mercapturic acid pathway. Following conjugation with GSH, the enzymatic actions of γ-glutamyl-transpeptidase and cysteinyl glycinase convert the dithiocarbamate to a cysteinyl glycine derivative and a cysteine derivative respectively (Keum et al. 2004).

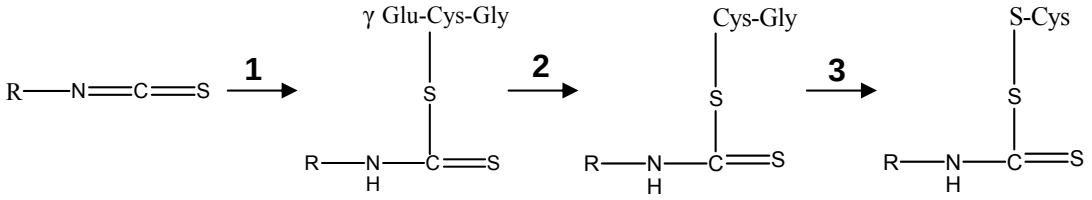


Figure 4.5: Formation of isothiocyanate-cysteine conjugates. *Intracellular isothiocyanates rapidly conjugate with GSH to form a dithiocarbamate* ***(1)****. The enzymatic activity of γ-glutamyl-transpeptidase then converts the dithiocarbamate to a cysteinyl glycine derivative* ***(2)****. Cysteinyl-glycinase then catalyses the conversion of the cysteinyl glycine derivative to a cysteine derivative* ***(3)****.*

Cysteine derivaties of isothiocyanates have been used in previous chemoprevention studies and may even be more clinically useful as they are proposed to be less toxic (Zheng et al. 1992). Two such cysteine derivatives, *S*-(*N*-benzylthiocarbamoyl)-*L*-cysteine, which is derived from benzyl isothiocyanate, and *S*-(*N*-3-phenylpropylthiocarbamoyl)-*L*-cysteine, which is derived from 3-phenylpropyl isothiocyanate, were examined for cytotoxic activity (Figure 4.6).

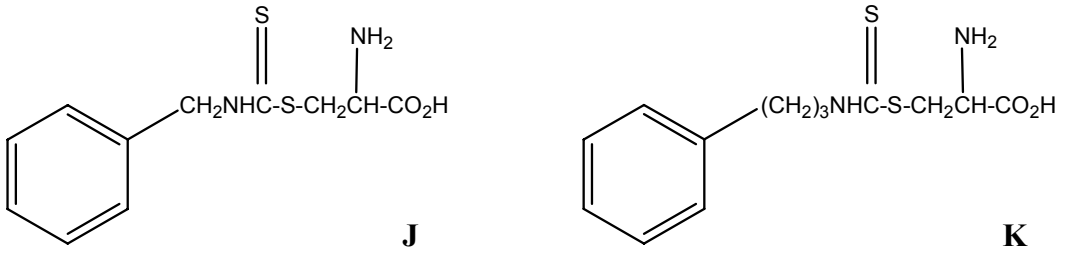


Figure 4.6: Structures of the products of aromatic isothiocyanate metabolism. (J) S-*(*N*-benzylthiocarbamoyl)*-L-*cysteine which is derived from benzyl isothiocyanate and* ***(K)*** S-*(*N*-3-Phenylpropylthiocarbamoyl)*-L-*cysteine which is derived from 3-phenylpropyl isothiocyanate.*

The cytotoxic activities of the isothiocyanate-cysteine conjugates (Figure 4.7) were greatly diminished when compared with their respective parent compounds (Figure 4.4B and 4.4D); however, high concentrations of *S*-(*N*-benzylthiocarbamoyl)-*L*-cysteine were able to induce complete loss of cell viability (Figure 4.7A). Although the cysteine derivatives were not as cytotoxic as the isothiocyanates that they are derived from, they did retain the ability to induce death of Bcl-2 overexpressing cells.

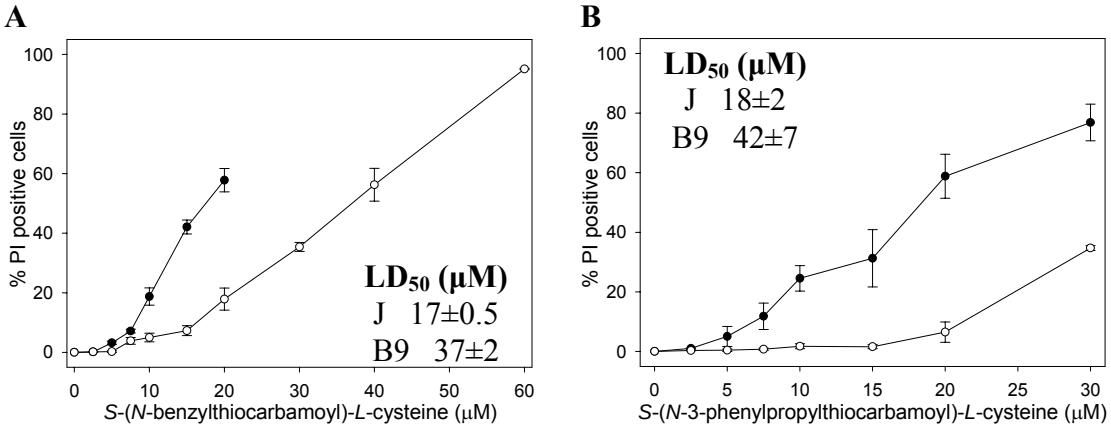


Figure 4.7: The cytotoxicity of* S-*(*N-*benzylthiocarbamoyl)*-L-*cysteine and* S-*(*N-*3-phenylpropylthiocarbamoyl)*-L-*cysteine. *Both Jurkat (●) and B9 (○) cells were treated for 24 hours with either* S-*(*N-*benzylthiocarbamoyl)*-L-*cysteine* ***(A)*** *or* S-*(*N-*3-phenylpropylthiocarbamoyl)*-L-*cysteine* ***(B)*** *before analysis of cell viability by flow cytometric determination of the uptake of PI (section 2.3.3). Plotted values represent the mean ± standard error of at least three experiments. LD_{50} values were generated using Sigmaplot Version 8.*

4.2.4 Structure-activity relationship of aliphatic isothiocyanates

Along with aromatic and indoyl isothiocyanates, a large number of naturally occurring aliphatic isothiocyanates have been identified. A selection of such compounds (Figure 4.8) was analysed to determine the efficacy of aliphatic isothiocyanates as cytotoxic compounds in the Bcl-2 overexpressing cells. Allyl isothiocyanate and sulforaphane (Figure 4.8M and 4.8Q) were of particular interest, as numerous studies have demonstrated the potent chemopreventive properties of these particular isothiocyanates (Conaway et al. 2002; Fimognari et al. 2002b; Munday and Munday 2004).

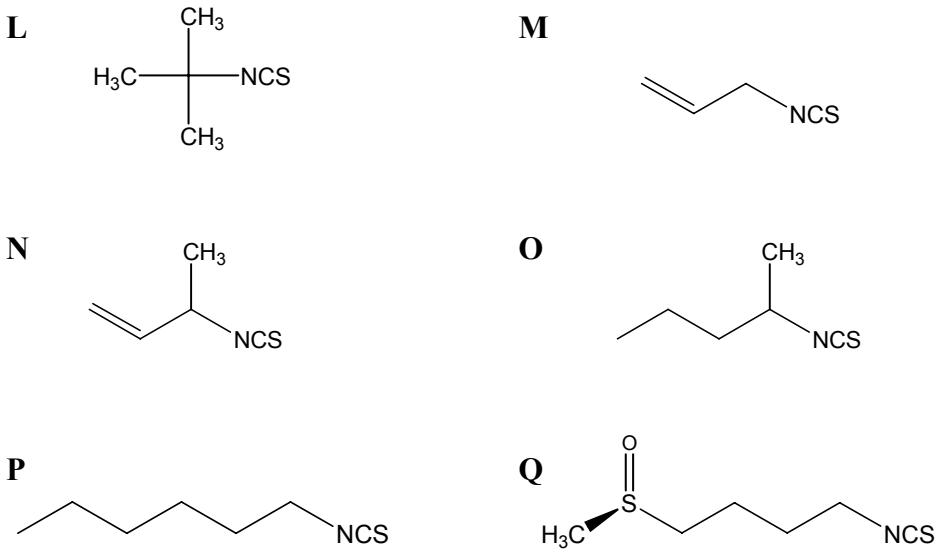


Figure 4.8: Structures of aliphatic isothiocyanates. (L) tert-*Butyl isothiocyanate, **(M)** allyl isothiocyanate, **(N)** 1-methyl allyl isothiocyanate, **(O)** 1-methyl butyl isothiocyanate, **(P)** hexyl isothiocyanate and **(Q)** sulforaphane [1-isothiocyanato-4-(methylsufinyl)-butane]*.

tert-Butyl isothiocyanate, 1-methyl allyl isothiocyanate, 1-methyl butyl isothiocyanate and hexyl isothiocyanate were completely devoid of any ability to induce cell death in the B9 cells (Figure 4.9A and 4.9C-E). Of these compounds, only hexyl isothiocyanate was able to appreciably affect the viability of the Jurkat cells; however, at 20 μM, greater than 80% of cells were still viable. At high concentrations, allyl isothiocyanate was cytotoxic to the Jurkat cells, but only induced a minimal amount of B9 cell death at concentrations as high as 60 μM (Figure 4.9B). The response of the B9 and Jurkat cells to sulforaphane was particularly interesting (Figure 4.9F). In all other dose-response curves, the isothiocyanates have demonstrated greater cytotoxicity towards the Jurkat cells than towards the B9 cells. Sulforaphane displayed a uniquely similar cell death profile in both cell lines.

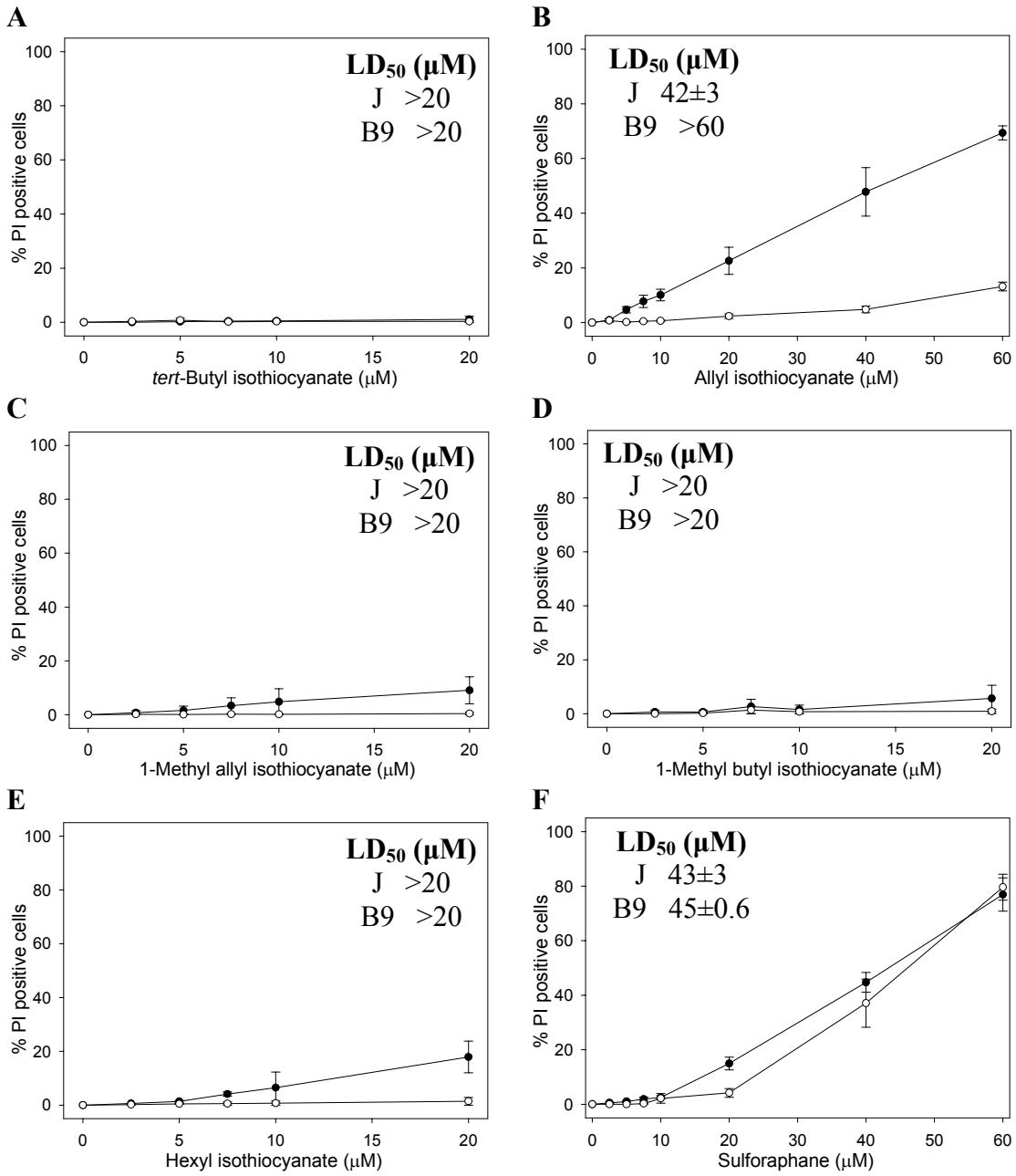


Figure 4.9: The cytotoxicity of aliphatic isothiocyanates*. Both Jurkat (●) and B9 (○) cells were treated for 24 hours with either* tert*-butyl isothiocyanate* ***(A)****, allyl isothiocyanate* ***(B)****, 1-methyl allyl isothiocyanate* ***(C)****, 1-methyl butyl isothiocyanate* ***(D)****, hexyl isothiocyanate* ***(E)*** *or sulforaphane* ***(F)*** *before analysis of cell viability by flow cytometric determination of the uptake of PI (section 2.3.3). Plotted values represent the means ± standard error of at least three experiments. LD_{50} values were generated using Sigmaplot Version 8.*

4.2.5 Isothiocyanates and sensitisation to Fas-mediated apoptosis

Bcl-2 overexpressing cells have previously been demonstrated to be resistant to the induction of Fas-mediated apoptosis (Kawahara et al. 1998), a property that can be reversed by addition of PEITC (Pullar et al. 2004). Due to the fact that Jurkat cells are

type II cells and rely on the cleavage of Bid and activation of the mitochondrial caspase-activation pathway, apoptosis induction in Jurkat cells is primarily mediated via the mitochondria (Cuvillier et al. 2000). Therefore, overexpression of Bcl-2 has a profound effect on the ability of the Fas ligand to induce apoptosis in Jurkat cells.

Various isothiocyanates were examined for their ability to sensitize the Bcl-2 overexpressing B9 cells to Fas-mediated apoptosis (Figure 4.10). Cells were treated with 15 μM of each aromatic isothiocyanate, corresponding to the LD_{50} of PEITC in B9 cells. Sulforaphane and allyl isothiocyanate were administered at a concentration of 40 μM, reflecting the LD_{50} of sulforaphane in B9 cells. Caspase activity was assessed after 4 hours, a time when PEITC alone had very little effect on caspase activation in B9 cells (section 3.3.5). Only PEITC and benzyl isothiocyanate (BITC) were able to sensitize the cells to Fas stimulation. Phenyl isothiocyanate did not affect the ability of the anti-Fas antibody to induce apoptosis. Sulforaphane (SFN) and allyl isothiocyanate (AITC) were also incapable of sensitising the Bcl-2 overexpressing cells to Fas-mediated apoptosis.

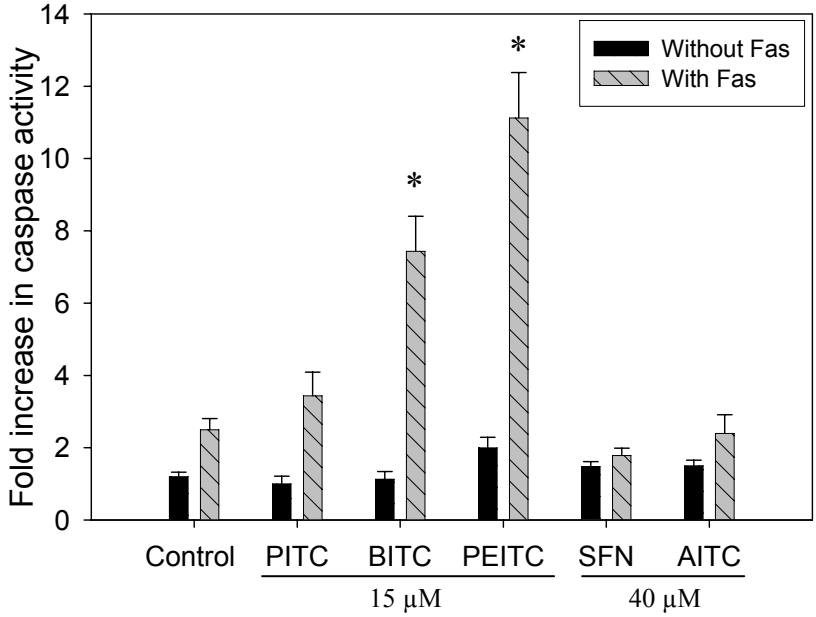


Figure 4.10: Sensitisation to Fas-mediated apoptosis by a selection of isothiocyanates. *B9 cells were treated for one hour with the appropriate isothiocyanate before 4 hours exposure to anti-Fas antibody. Caspase activity was analysed by measuring the release of AMC from the artificial caspase substrate Ac-DEVD-AMC (section 2.3.5). Plotted values represent the mean ± standard error of at least three experiments. * Indicates a significant difference ($P<0.05$) from control cells treated with Fas (One Way Repeated Measures ANOVA with Bonferroni multiple comparison [SigmaStat]).*

4.2.6 The antiproliferative activity of various isothiocyanates

Due to the fact that inhibition of proliferation is tightly linked with apoptosis and loss of cell viability, the anti-proliferative activities of a selection of isothiocyanates were determined. Screening of the ability of the isothiocyanates to induce cell death identified differential potencies, depending on structure. However, it is possible that an isothiocyanate may be able to inhibit proliferation, but not proceed to induce cell death.

Both aliphatic and aromatic isothiocyanates were shown to be capable of inhibiting Jurkat cell proliferation, but to varying degrees (Figure 4.11 and Figure 4.12). The aromatic isothiocyanates (Figure 4.12) displayed more potent anti-proliferative activity than the aliphatic isothiocyanates (Figure 4.11). The only exception was phenyl isothiocyanate which demonstrated the weakest anti-proliferative activity and only reduced proliferation to a maximum of 20%. The strongest anti-proliferative compounds were PEITC and benzyl isothiocyanate with IC_{50} values of 3.2 µM and 3.5 µM respectively. The products of aromatic isothiocyanate metabolism (Figure 4.13) demonstrated weaker anti-proliferative activity than their respective parental compounds (Figure 4.12B and 4.12D); however, they still dramatically reduced the proliferation of Jurkat cells after 1 hour of exposure.

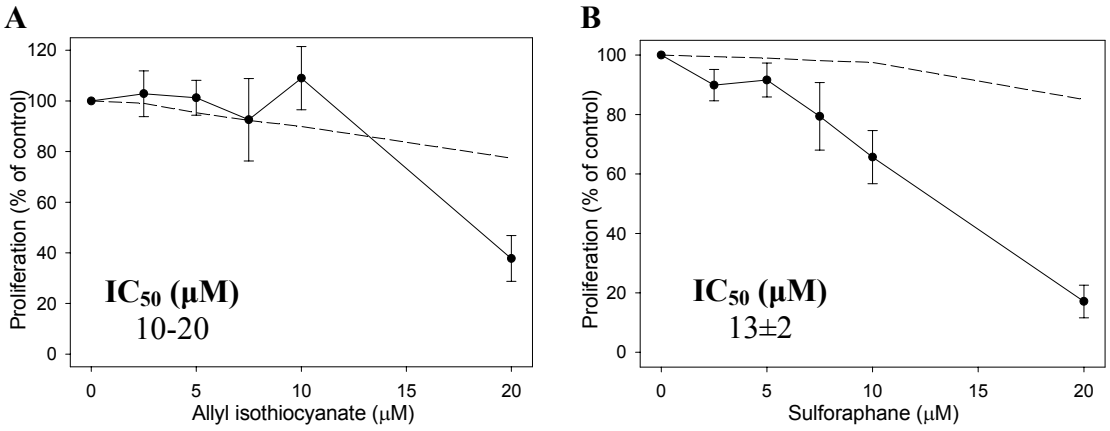


Figure 4.11: The antiproliferative activity of aliphatic isothiocyanates. *Jurkat cells were treated for one hour with* ***(A)*** *allyl isothiocyanate, or* ***(B)*** *sulforaphane. Cell proliferation was then measured by monitoring the incorporation of BrdU into the DNA of proliferating cells using an ELISA (section 2.3.2). Plotted values represent mean ± standard error of at least three experiments and are expressed as a percentage of the proliferation observed in untreated cells. Dose-response curves for loss of cell viability are overlayed in each graph (dashed line).*

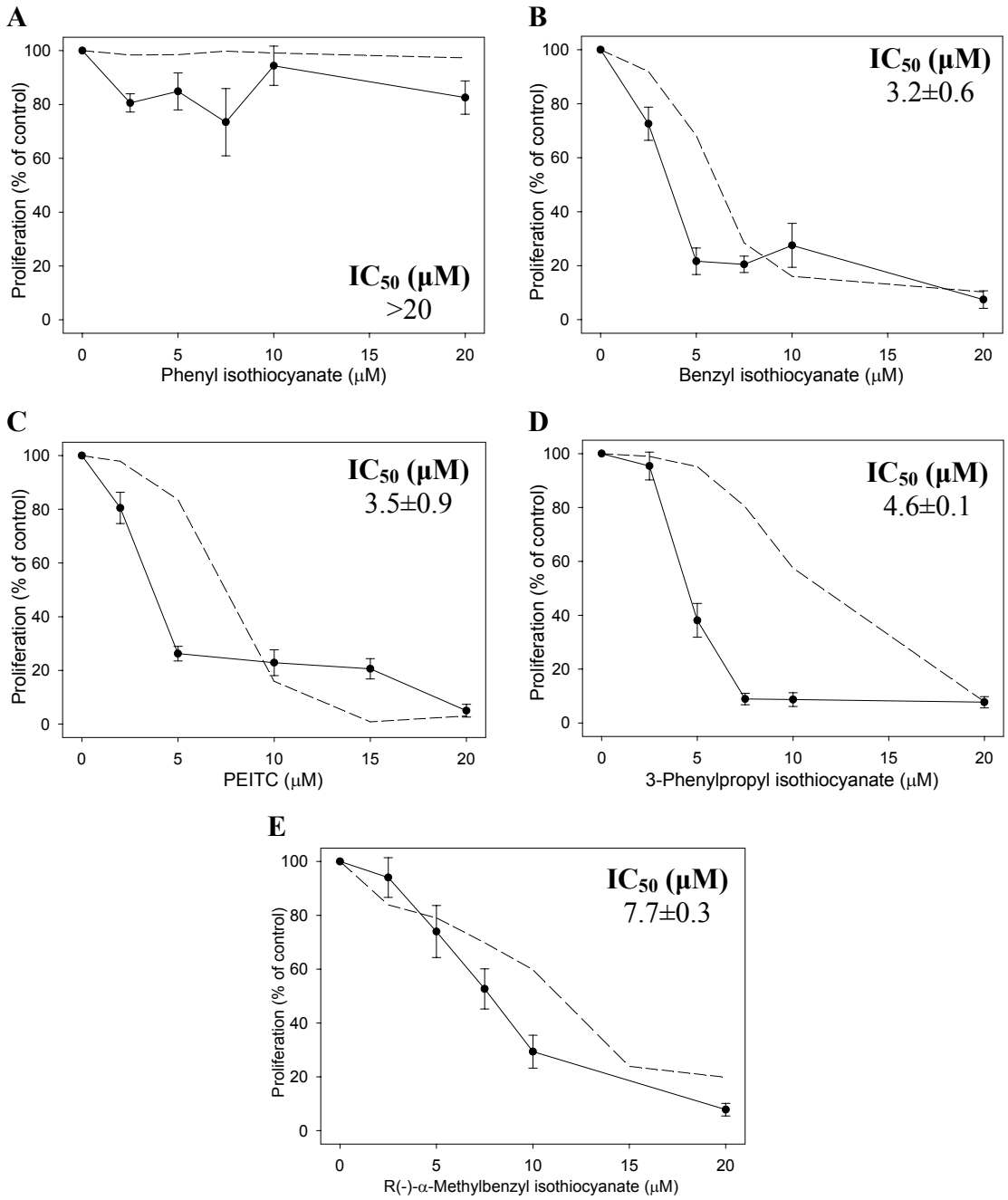


Figure 4.12: The antiproliferative activity of aromatic isothiocyanates. *Jurkat cells were treated for one hour with* ***(A)*** *phenyl isothiocyanate,* ***(B)*** *benzyl isothiocyanate,* ***(C)*** *PEITC,* ***(D)*** *3-phenylpropyl isothiocyanate, or* ***(E)*** *R(-)-α-methylbenzyl isothiocyanate. Cell proliferation was then measured by monitoring the incorporation of BrdU into the DNA of proliferating cells using an ELISA (section 2.3.2). Plotted values represent mean ± standard error of at least three experiments and are expressed as a percentage of the proliferation observed in untreated cells. Dose-response curves for loss of cell viability are overlayed in each graph (dashed line).*

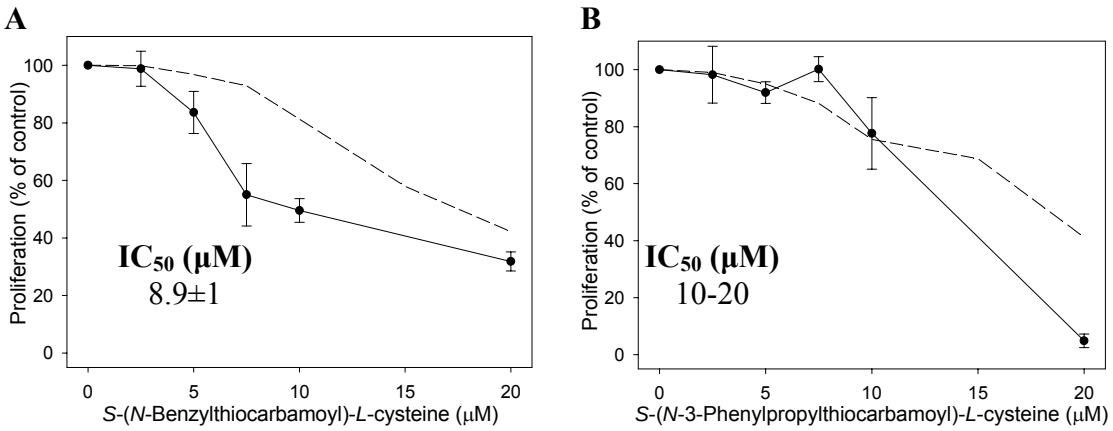


Figure 4.13: The antiproliferative activity of products of aromatic isothiocyanate metabolism. *Jurkat cells were treated for one hour with (A)* S-*(*N-*benzylthiocarbamoyl)*-L-*cysteine, or (B)* S-*(*N-*3-phenylpropylthiocarbamoyl)*-L-*cysteine. Cell proliferation was then measured by monitoring the incorporation of BrdU into the DNA of proliferating cells using an ELISA (section 2.3.2). Plotted values represent mean ± standard error of at least three experiments and are expressed as a percentage of the proliferation observed in untreated cells. Dose-response curves for loss of cell viability are overlayed in each graph (dashed line).*

A relationship between anti-proliferative activity and ability to induce loss of cell viability was observed (Figure 4.14). A statistically significant correlation between the LD_{50} values for loss of cell viability in Jurkat cells and IC_{50} values for inhibition of proliferation was observed (Pearson correlation, P=0.02).

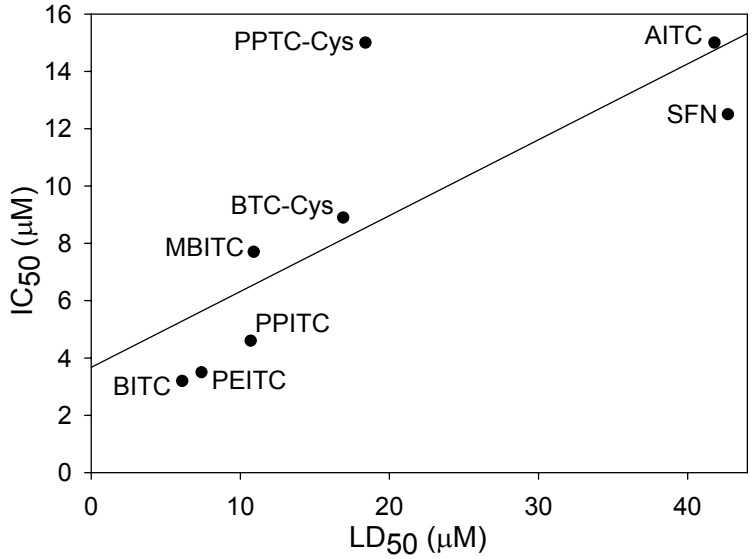


Figure 4.14: Correlation between LD_{50} and IC_{50} values for different isothiocyanates. *BITC=benzyl isothiocyanate, PPITC=3-phenylpropyl isothiocyanate, MBITC=R(-)-α-methylbenzyl isothiocyanate, BTC-Cys=S-(*N-*benzylthiocarbamoyl)*-L-*cysteine, AITC=allyl isothiocyanate, SFN=sulforaphane, PPTC-Cys=S-(*N-*3-phenylpropylthiocarbamoyl)*-L-*cysteine. r^2=0.794, P=0.02 (Sigmastat, Jandel Scientific).*

4.2.7 The cytotoxic and anti-proliferative activities of various isothiocyanates

Isothiocyanate	Cell Viability LD_{50} (μM)		Proliferation IC_{50} (μM)
	Jurkat	B9	Jurkat
Benzyl thiocyanate	> 60	> 60	
Benzyl selenocyanate	11 ± 0.4	18 ± 0.2	
Phenyl isothiocyanate	> 60	> 60	>20
Benzyl isothiocyanate	6.1 ± 0.1	23 ± 6	3.2 ± 0.6
Phenethyl isothiocyanate	7.4 ± 0.1	15 ± 0.2	3.5 ± 0.9
3-Phenylpropyl isothiocyanate	11 ± 0.1	21 ± 0.3	4.6 ± 0.1
4-Phenylbutyl isothiocyanate	10 ± 2	23 ± 0.2	
Phenylhexyl isothiocyanate	11 ± 0.6	25 ± 0.3	
R(-)-α-Methylbenzyl isothiocyanate	11 ± 2	32 ± 2	7.7 ± 0.3
S-(N-benzylthiocarbamoyl)-L-cysteine	17 ± 0.5	37 ± 2	8.9 ± 1
S-(N-3-phenylpropylthiocarbamoyl)-L-cysteine	18 ± 2	42 ± 7	10-20
t-Butyl isothiocyanate	>20	> 20	
Allyl isothiocyanate	42 ± 3	> 60	10-20
1-Methyl allyl isothiocyanate	> 20	> 20	
1-Methyl butyl isothiocyanate	> 20	> 20	
Hexyl isothiocyanate	> 20	> 20	
Sulforaphane	43 ± 3	45 ± 0.6	13 ± 2

Table 4.1: Summary of isothiocyanate concentrations required to induce 50% loss of cell viability (LD_{50}) in Jurkat and B9 cells and 50% inhibition of proliferation (IC_{50}) in Jurkat cells*. LD_{50} and IC_{50} values were generated using Sigmaplot Version 8 based on replicate data from Figures 4.2, 4.4, 4.7, and 4.9 for cell viability and Figures 4.11, 4.12 and 4.13 for proliferation. All values represent the mean ± standard error of at least three experiments.*

4.3 Discussion

This investigation represents the first large scale screening of isothiocyanates for chemotherapeutic potential. The cytotoxic activities of aromatic isothiocyanates, products of aromatic isothiocyanate metabolism and aliphatic isothiocyanates were investigated. This is also the first time that a selection of isothiocyanates has been screened for cytotoxic activity towards drug-resistant Bcl-2 overexpressing cells.

Results from this section of the study highlight the critical role that intrinsic structural differences of various isothiocyanates play in affording biological activity towards cells that overexpress Bcl-2. PEITC displayed the most potent activity in inducing loss of cell viability, inhibiting proliferation and sensitising Bcl-2 overexpressing cells to Fas-mediated apoptosis. Benzyl isothiocyanate was the other most promising compound identified. Previous studies have also identified PEITC and benzyl

isothiocyanate as two extremely promising compounds with respect to the ability to induce apoptotic cell death (Jakubikova et al. 2005; Tang and Zhang 2005).

The isothiocyanate moiety was clearly identified as being absolutely required for cytotoxic activity in our system. The importance of the isothiocyanate group was also recognised in a study comparing the inhibition of mitochondrial respiration by benzyl isothiocyanate (BITC) and the methyl-thiocarbamate analogue of benzyl isothiocyanate (BITC-OMe) (Kawakami et al. 2005). While BITC potently inhibitied respiration, BITC-OMe displayed no activity. Due to the greater electrophilicity of the carbon between the nitrogen and sulfur, benzyl isothiocyanate can act as an electrophilic trapping agent for a nucleophile derived from a biological target, such as a protein (personal communication with Prof. Margaret Brimble, Department of Chemistry, University of Auckland). Given that isothiocyanate reactivity confers biological activity, in our system the reactions of cellular nucleophiles with the electrophilic isothiocyanate moiety is implicated in the induction of cell death in cells overexpressing Bcl-2.

The side chain attached to the isothiocyanate group also profoundly influenced cytotoxic and anti-proliferative activity. Generally, the aromatic isothiocyanates were more potent than the aliphatic isothiocyanates in all three biological activities assessed. The only aromatic isothiocyanate that showed a complete absence of activity was phenyl isothiocyanate. The lack of a carbon spacer group between the aromatic ring and the isothiocyanate moiety rendered phenyl isothiocyanate completely ineffective at inducing cell death and inhibiting proliferation. The inactivity of phenyl isothiocyanate has been observed in previous studies with significant cytotoxicity only presenting at concentrations in excess of 700 μM (Lui et al. 2003). Addition of a single CH_2 unit, giving rise to benzyl isothiocyanate, was sufficient to confer strong cytotoxic and anti-proliferative activity in both the Jurkat and B9 cells. The absence of a carbon spacer group promotes electron withdrawal by the phenyl ring which increases the electrophilicity of the central carbon of the isothiocyanate moiety. The increased reactivity of phenyl isothiocyanate means that it could react with constituents in the culture media or react with other cellular proteins before reaching the intracellular site(s) critical for promotion of apoptosis (personal communication with Dr Yuesheng Zhang, Roswell Park Cancer Institute). This is

supported by studies which have shown that phenyl isothiocyanate is inactive in assays using cultured cell systems but displays inhibitory activity when used to inhibit the respiration of isolated mitochondria (Zhang and Talalay 1998; Kawakami et al. 2005). However, when either the Jurkat or B9 cells were suspended in PBS, phenyl isothiocyanate remained inactive (data not shown), suggesting that phenyl isothiocyanate will react with GSH or other non-essential thiols in cells.

Studies on the inhibition of the enzyme papain by various isothiocyanates identified an alternate explanation for differences in biological activity and provides an interesting perspective as to why the small structural differences between phenyl isothiocyanate and BITC affect biological activity (Tang and Tang 1976). A large number of isothiocyanates with diverse structural characteristics were examined but BITC was identified as the lead compound. The authors proposed formation of a thiocarbamic linkage between the active site sulfhydryl group of papain and the isothiocyanate moiety of benzyl isothiocyanate as the mechanism for inhibitory activity. The sulfhydryl residue of papain exists in the vicinity of an aromatic binding site which is hypothesised to interact with the aromatic ring of BITC. The isothiocyanate moiety can then be aligned for nucleophilic attack by the carbon linker group; therefore, the length of the linker group plays a role in inhibitory activity.

The target molecules in our system may be modified by a similar reaction and the structural features of an isothiocyanate may influence inhibitory or activating potential in a similar fashion. This will only become apparent upon identification of the primary target(s) responsible for enabling apoptosis in the cells overexpressing Bcl-2. Chapter Five outlines our initial investigations to address this issue.

Chapter 5

An investigation of the molecular targets of the isothiocyanates

5.1 Introduction

A number of proteins that are involved in cell signalling pathways, including kinases, phosphatases and transcription factors, contain critical thiol residues. Oxidation of such thiol residues can dramatically alter the function of a protein by disrupting its structure and conformation, or by directly inhibiting catalytic activity (Saitoh et al. 1998; Korn et al. 2001; Buzek et al. 2002; Chiarugi and Cirri 2003; Sun et al. 2003; Cross and Templeton 2004a). Due to the dramatic consequences of such a modification, thiol oxidation is believed to play a critical role in controlling the equilibrium between cell survival and induction of apoptosis by activating proapoptotic signals or by inhibiting cell survival pathways (Cross and Templeton 2004b; Biswas et al. 2005).

Upon entering a cell, an isothiocyanate can participate in two principal reactions, both involving intracellular thiol-containing molecules. Firstly, glutathione *S*-transferases catalyse the rapid conjugation of intracellular isothiocyanates with the cells most abundant thiol-containing molecule, glutathione (GSH) (Zhang et al. 1995). Alternatively, intracellular isothiocyanates can react with protein thiol residues to form dithiocarbamate derivatives (Zhang et al. 2006) (Figure 5.1).

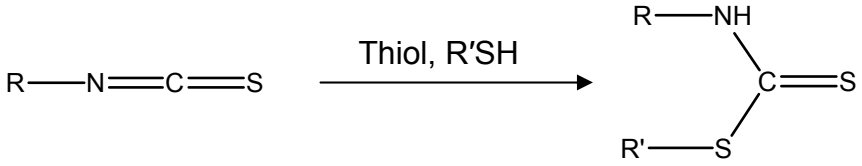


Figure 5.1: Reaction of an isothiocyanate with a protein thiol residue. *The reaction of an isothiocyanate with a protein thiol (R'SH) results in the formation of a dithiocarbamate derivative (adapted from Thornalley 2002).*

The intracellular reactions of the isothiocyanates have the potential to promote indirect oxidation of thiol proteins by decreasing intracellular levels of GSH (Xu and Thornalley 2001a), and by inactivation of other cellular reductants, thereby promoting an oxidising environment. A decline in cellular reductants would allow accumulation of thiol proteins oxidised by endogenous reactive oxygen species (ROS) produced during normal cell metabolism (Balaban et al. 2005). Generation of ROS and loss of GSH also occur as a consequence of apoptosis (van den Dobbelsteen et al. 1996; Macho et al. 1997; Cai and Jones 1998); however, we are interested in the early stages of apoptosis and initial targets of the isothiocyanates that are modified, well before activation of the apoptotic pathway.

It seems extremely likely that the mechanism by which the isothiocyanates promote apoptosis in cells is due to their ability to alter protein function through indirect oxidative modification of thiol residues or direct modification of thiol proteins, via the formation of dithiocarbamates. Therefore, the aim of this section of the study was to identify global changes in oxidised thiol proteins in response to isothiocyanate treatment.

PEITC and sulforaphane were chosen as the compounds of interest. The isothiocyanate of most interest was PEITC, which was identified as having the greatest potential to induce apoptosis in cells that overexpress Bcl-2 (Chapter Three and section 4.2.7). On the other hand, sulforaphane was not capable of killing Bcl-2 overexpressing cells unless administered at high concentrations (section 4.2.4). Furthermore, at high concentrations, sulforaphane did not sensitize cells to Fas-mediated apoptosis (section 4.2.5), suggesting that it is incapable of overcoming the anti-apoptotic block that overexpression of Bcl-2 confers. However, sulforaphane is known to potently modulate the activities of some phase I and phase II enzymes through modification of thiol proteins (Hong et al. 2005a). By comparing the response of B9 cells to PEITC with the response of B9 cells to sulforaphane, the goal is to distinguish between proteins whose thiol residues are oxidised as a general response to isothiocyanate treatment (and therefore should change with both PEITC and sulforaphane), or, specifically, due to treatment with PEITC. By utilising the two isothiocyanates, the identification of specific proteins that may play a crucial role in the induction of apoptosis by PEITC can be identified.

5.2 Experimental approach

Most intracellular protein thiol groups exist in a reduced form (Schafer and Buettner 2001); therefore, it is easier to detect an increase in oxidised thiol proteins rather than a loss of reduced thiol proteins. Oxidised thiol proteins were monitored by utilising a sensitive proteomic technique previously developed in our laboratory (Baty et al. 2002).

Initially, cells are treated with an isothiocyanate for 30 minutes before addition of N-ethylmaleimide (NEM), which blocks the remaining reduced thiol residues (Figure 5.2). Cells are then lysed, and the oxidised thiols reduced with dithiothreitol (DTT), before addition of the thiol-specific fluorescent probe 5-iodoacetamidofluorescein (IAF).

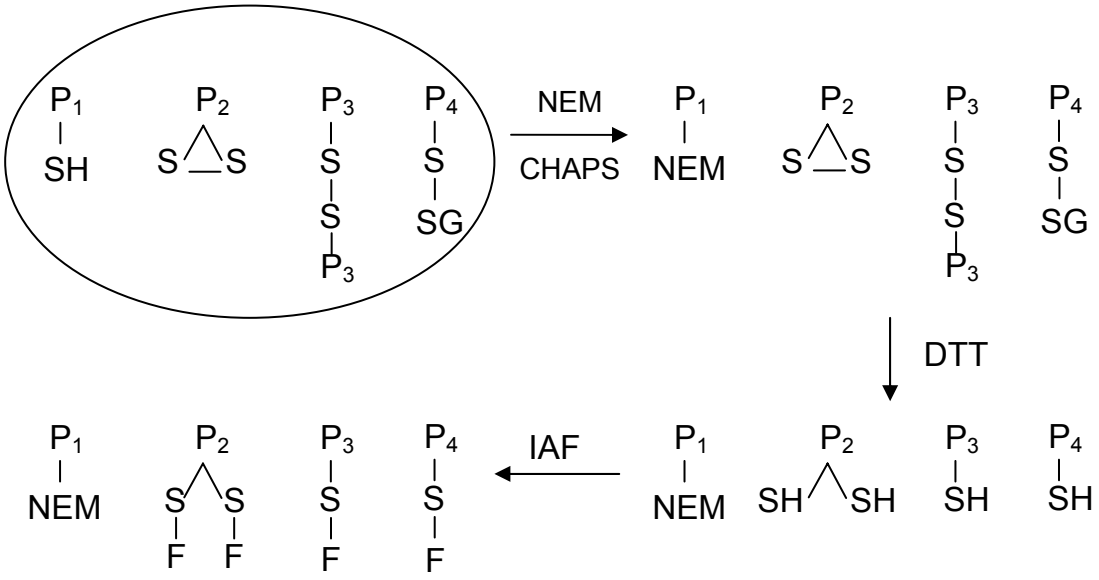


Figure 5.2: Fluorescent labelling of oxidised thiol proteins. *Whole cells are incubated with NEM to block reduced thiols (P_1) before being lysed with CHAPS. DTT is then added to reduce oxidised thiols (P_{2-4}) which are then labelled with IAF (P-S-F). P_1SH represents reduced thiol proteins, P_2S-S represents intramolecular disulfides, P_3S-SP_3 represents intermolecular disulfides and P_3S-SG represents a mixed disulfide with glutathione (Baty et al. 2002).*

The resulting protein lysates are separated by two-dimensional (2-D) electrophoresis, which separates proteins according to their respective isoelectric points and molecular weight values (Garfin 2003). Fluorescence scanning of the resulting gels enables the visualisation of the cellular complement of oxidised thiol proteins and changes to the fluorescence intensity of individual proteins in response to treatment with the

isothiocyanates. Three independent experiments were performed and only thiol proteins with a consistent two-fold increase or decrease in IAF fluorescence intensity in at least two experiments were selected.

5.3 Results

Oxidation of protein thiol residues in response to isothiocyanate exposure was examined in the Bcl-2 overexpressing B9 cells. B9 cells were treated with 15 µM of either PEITC or sulforaphane, corresponding to the LD_{50} for PEITC in B9 cells (section 3.3.4) (Figure 5.3A). At this concentration, sulforaphane kills less than 5% of B9 cells (section 4.2.4). Cells were treated for 30 minutes, a time point long before PEITC can trigger caspase activation and induce apoptosis in B9 cells (section 3.3.5) (Figure 5.3B). Therefore, any differences to IAF labelling of thiol proteins represent very early changes, occurring upstream of activation of the apoptotic pathway.

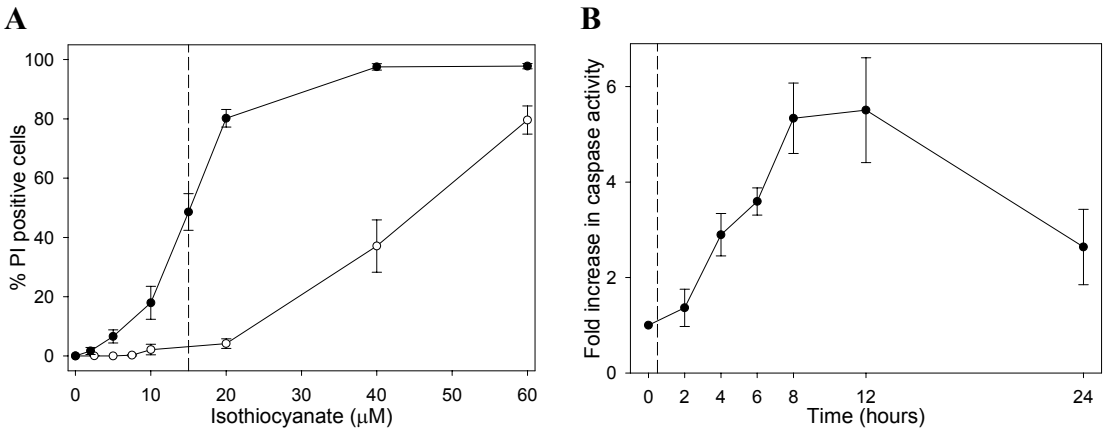


Figure 5.3: Loss of B9 cell viability in response to treatment with PEITC or sulforaphane and the time course of B9 caspase activity in response to 15 µM PEITC. (A) *B9 cells were treated with PEITC (●) or sulforaphane (○) for 24 hours before assessment of cell viability by flow cytometric determination of the uptake of PI (section 2.3.3). Plotted values represent mean ± standard error of at least three experiments.* ***(B)*** *B9 cells were treated with 15 µM PEITC and samples were removed for caspase activity analysis 2, 4, 6, 8, 12 and 24 hours after treatment. Caspase activity was monitored by measuring the release of AMC from the artificial caspase substrate Ac-DEVD-AMC (section 2.3.4). Plotted values represent mean ± standard error of at least four experiments. Caspase activity is expressed as a fold increase over the caspase activity that could be detected in control cells.*

The pattern of fluorescent labelling was quite different from the pattern of total protein distribution, as determined by a silver stain (Figure 5.4). A large number of proteins easily visible following silver staining were not detected by fluorescence

scanning (Figure 5.4, Box 1). Conversely, regions with no visible silver staining were clearly visible when imaged for IAF-labelled oxidised thiol proteins (Figure 5.4, Box 2). The IAF-labelling protocol can therefore permit visualisation of changes to low abundance thiol proteins.

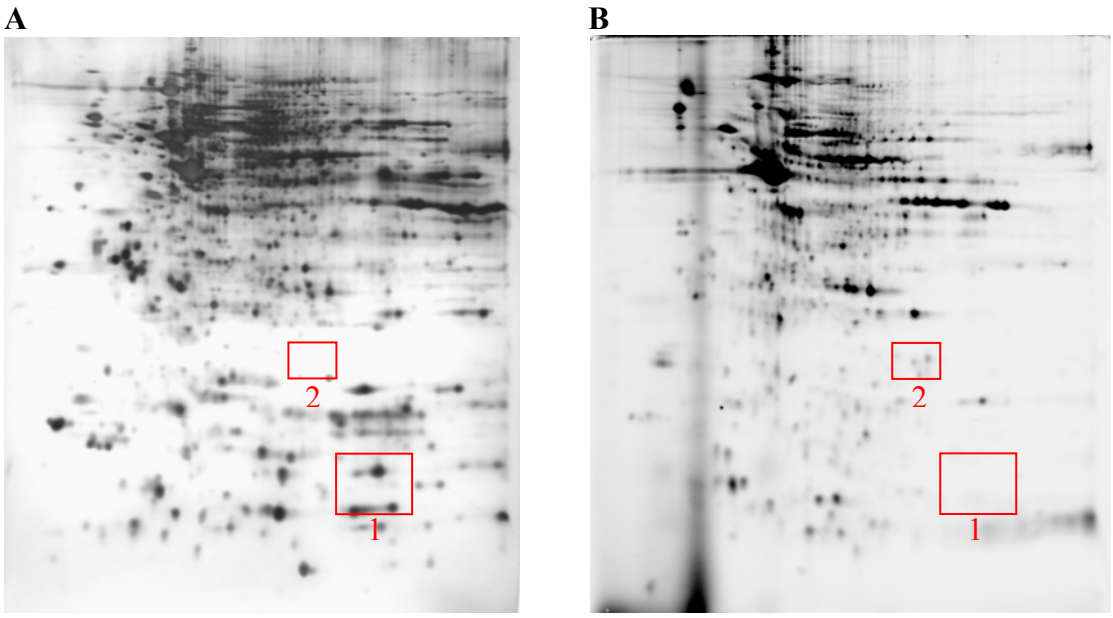


Figure 5.4: Silver stain and IAF fluorescence labelling of untreated B9 cells. *An untreated B9 protein lysate was analysed by 2-D electophoresis (section 2.3.8). (A) A representative silver stain (section 2.3.10) of total protein content and (B) the corresponding fluorescence scan for IAF-labelled oxidised thiol proteins. A region of high protein content but no IAF fluorescence is highlighted in Box 1. A region of low protein content but visible IAF fluorescence is highlighted in Box 2. Images are representative of gels from three experiments.*

The pattern of thiol oxidation following exposure to PEITC was also very different to that observed following treatment with the oxidant hydrogen peroxide (H_2O_2) (Baty et al. 2005). Most notably there was no change in the oxidation state of the various isoforms of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in response to isothiocyanate exposure (Figure 5.5, Box 1). GAPDH is a thiol-containing protein that is particularly sensitive to oxidation and a strong increase in IAF labelling of GAPDH has been observed when cells are treated with 20 µM H_2O_2 (Baty et al. 2005).

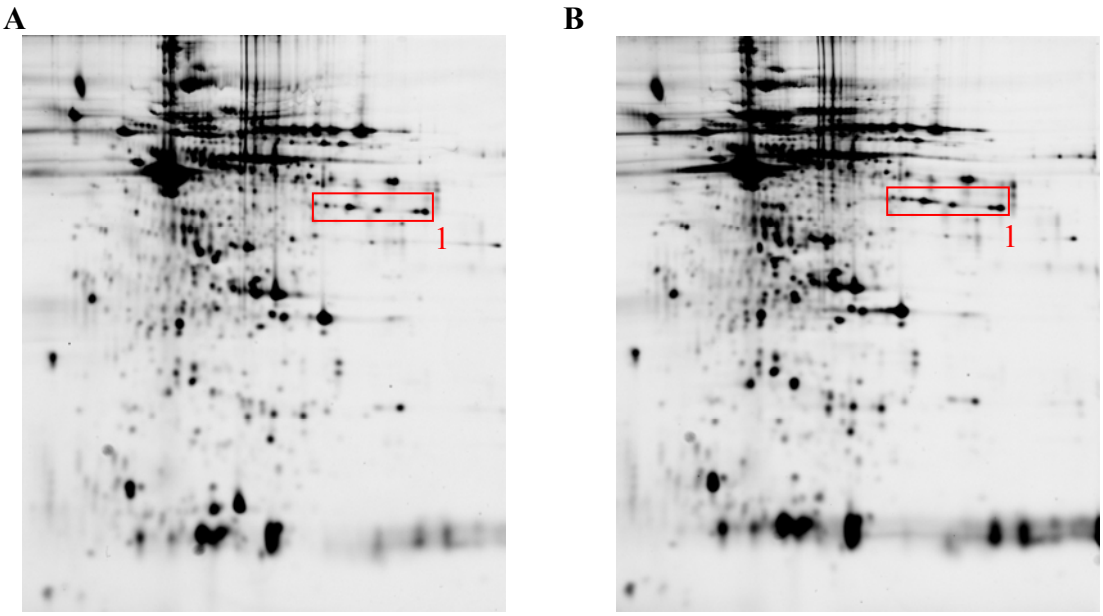


Figure 5.5: Pattern of IAF fluorescence labelling of untreated B9 cells and B9 cells treated with 15µM PEITC. *B9 cells were either left untreated* ***(A)*** *or treated with 15 µM PEITC for 30 minutes* ***(B)*** *before labelling of oxidised thiol proteins with IAF. IAF-labelled samples were analysed by two-dimensional electrophoresis (section 2.3.8). Images are representative of gels from three experiments. GAPDH isoforms are highlighted in Box 1.*

Changes to the intensity of IAF labelling of individual proteins were analysed with PDQuest™ 2-D electrophoresis gel analysis software (version 7.1.1, Bio-Rad Laboratories, Hercules, CA, USA) (section 2.3.9). Three separate experiments were performed and only thiol proteins with a two-fold increase or decrease in fluorescence in at least two of the three experiments were selected. A total of 390 thiol protein spots were detected following IAF labelling of B9 cells. A comparison of untreated B9 cells with PEITC-treated B9 cells revealed 35 spots with consistent changes to fluorescence intensity following exposure to PEITC (Figure 5.6 and zoom images for each spot in the appendix). The fluorescence intensities of 11 thiol proteins (31%) decreased as a result of exposure to PEITC (Figure 5.6A). The majority of thiol proteins altered in response to treatment with PEITC demonstrated an increase in the degree of IAF fluorescence (Figure 5.6B). A consistent increase in fluorescence was observed for 24 of the 35 spots (69%).

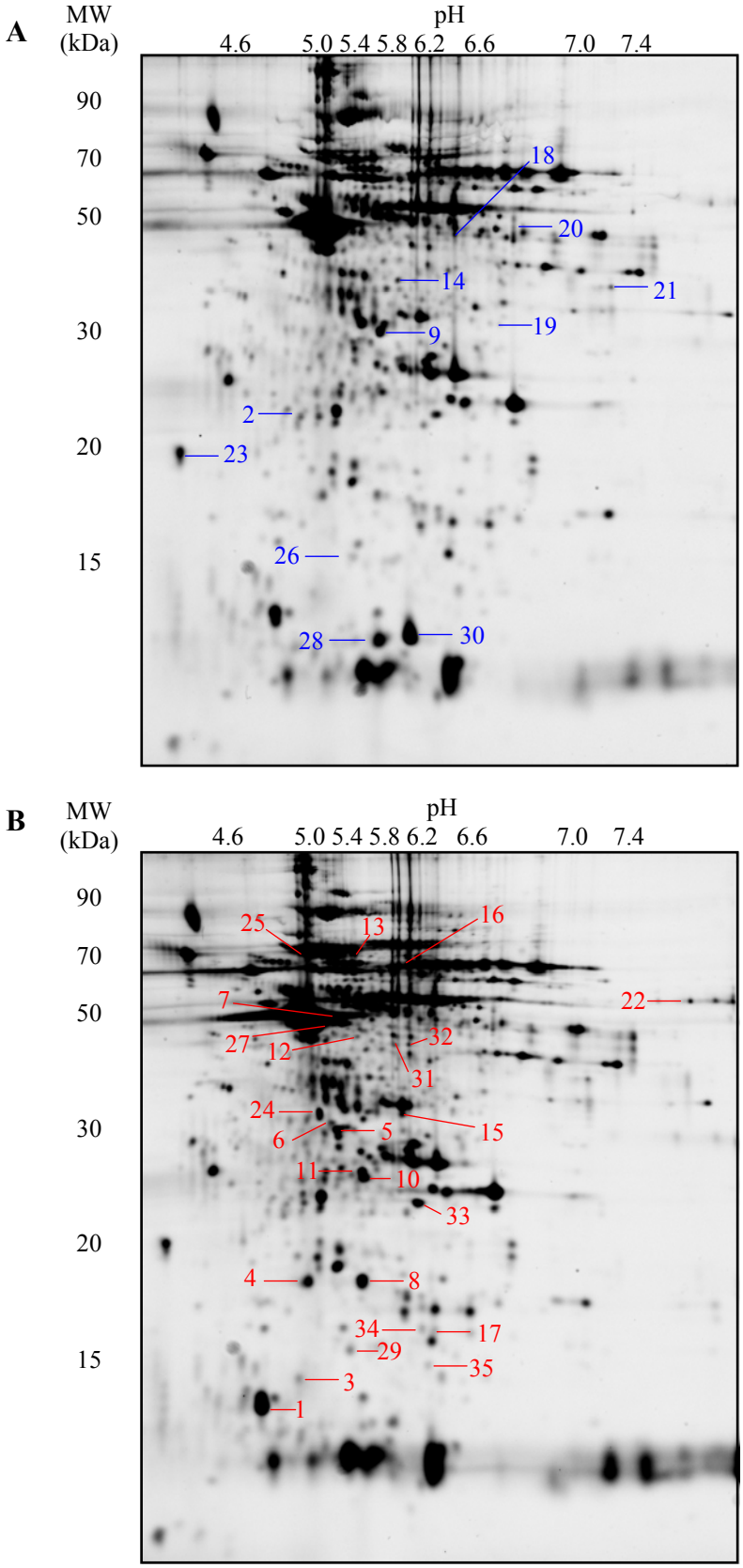


Figure 5.6: Oxidation of protein thiols following exposure to PEITC. *Untreated B9 cells* ***(A)*** *and B9 cells treated with 15 µM PEITC for 30 minutes* ***(B)****. Protein lysates were separated by 2-D electrophoresis (section 2.3.8). Gels are representative of three experiments. Spots with a two-fold decrease in fluorescence are highlighted in (A) while spots with a two-fold increase in fluorescence are highlighted in (B).*

The 35 spots that changed with PEITC were compared with the location of proteins previously identified in our laboratory as having changed following H_2O_2 treatment (Baty et al. 2005). In this earlier work, protein spots were identified by peptide mass fingerprinting using MALDI-TOF MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry). Briefly, spots were excised from gels and treated with trypsin to digest the protein. The peptides were then eluted and analysed by MALDI-TOF MS. The resulting peptide mass fingerprints, estimated molecular weight (MW) and isoelectric point (pI) values were then used to search online protein databases to identify the excised protein (Beranova-Giorgianni 2003).

The only thiol protein that was similar to thiol protein changes observed in previous experiments (Baty et al. 2005), constituted spot number 10 and spot number 11 (Figure 5.7). Spot number 10, with a molecular weight of 26.1 kDa and isoelectric point of 5.5, and spot number 11, with a molecular weight of 26.6 kDa and isoelectric point of 5.5 (Appendix) represent peroxiredoxin-3. A consistent increase in the fluorescence intensity of this thiol protein was observed when B9 cells were treated with PEITC (Figure 5.7B). A change in the extent of IAF labelling of spot number 10 or 11 was not observed when B9 cells were treated with sulforaphane (Figure 5.7C).

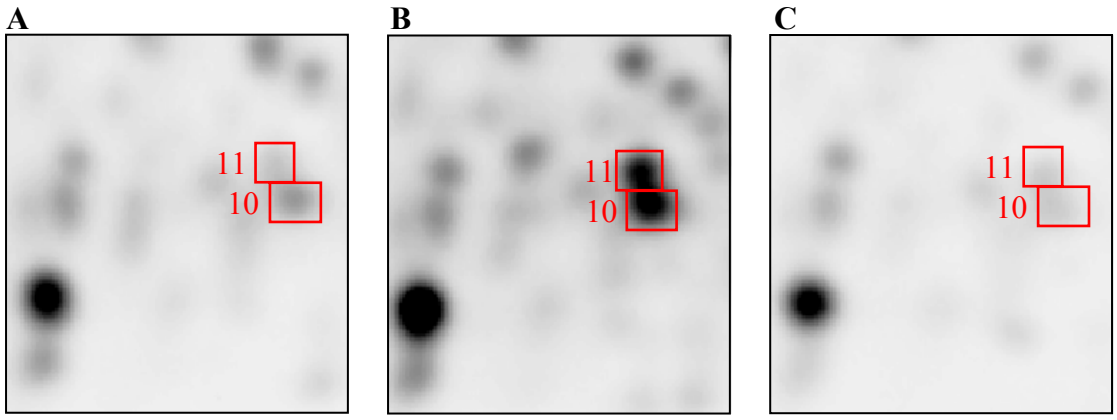


Figure 5.7: Changes in IAF-labelling of spot number 10 and spot number 11 in response to treatment with 15 µM PEITC but not 15 µM sulforaphane. *Fluorescence intensity of spot number 10 (MW=26.1, pI=5.5) and spot number 11 (MW=26.6, pI=5.5) in untreated B9 cells* ***(A)****, B9 cells exposed to 15 µM PEITC for 30 minutes* ***(B)*** *and B9 cells exposed to 15 µM sulforaphane for 30 minutes* ***(C)****. Shown are zoom images of spot number 10 and 11 from Figure 5.6 and Figure 5.8.*

A comparison of untreated B9 cells with sulforaphane-treated B9 cells revealed 27 thiol proteins with a consistent two-fold increase or decrease in fluorescence intensity (Figure 5.8 and zoom images for each spot in the appendix). Thirteen of the 27 thiol proteins (spot numbers 23-35) were also found to consistently change when B9 cells were treated with PEITC. The fluorescence of the remaining 14 thiol proteins (spot numbers 36-49) changed exclusively in response to treatment with sulforaphane. Fourteen of the 27 (52%) thiol proteins altered in response to exposure to sulforaphane experienced a decrease in the degree of IAF labelling (Figure 5.8A). A consistent increase in fluorescence was observed for the remaining 13 spots (48%) (Figure 5.8B).

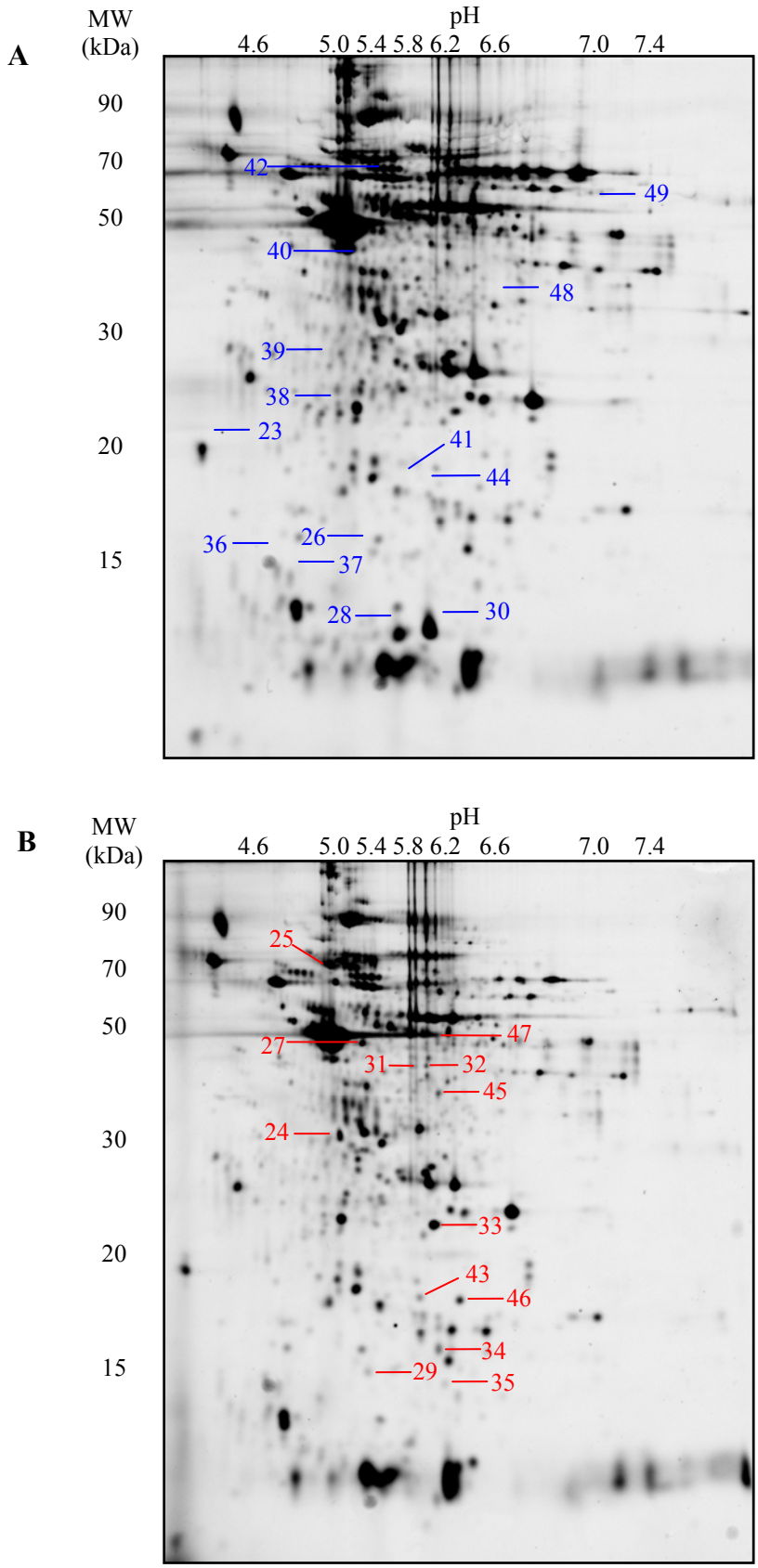


Figure 5.8: Oxidation of protein thiols following exposure to sulforaphane. *Untreated B9 cells* ***(A)*** *and B9 cells treated with 15 μM sulforaphane for 30 minutes* ***(B)****. Protein lysates were separated by 2-D electrophoresis (section 2.3.8). Gels are representative of three experiments. Spots with a two-fold decrease in fluorescence are highlighted in (A) while spots with a two-fold increase in fluorescence are highlighted in (B).*

In summary, 49 thiol proteins were modified following exposure to either PEITC or sulforaphane (Figure 5.9). Thirty-five protein thiol spots consistently changed in response to exposure to PEITC, whereas 27 thiol proteins consistently changed in response to exposure to sulforaphane. Thirteen of the 27 spots that changed with sulforaphane were also observed to change when B9 cells were treated with PEITC. Overall, the fluorescence intensities of 21 of the 49 spots (43%) decreased with isothiocyanate treatment. The remaining 28 thiol proteins (57%) demonstrated an increase in fluorescence. A large majority of thiol proteins altered in response to both sulforaphane and PEITC treatment, or in response to PEITC treatment only, demonstrated an increase in the degree of IAF labelling (69% and 68% respectively). In contrast, only 29% of thiol proteins that were modified by exposure to sulforaphane demonstrated an increase in fluorescence with most of the spots (71%) decreasing in fluorescence.

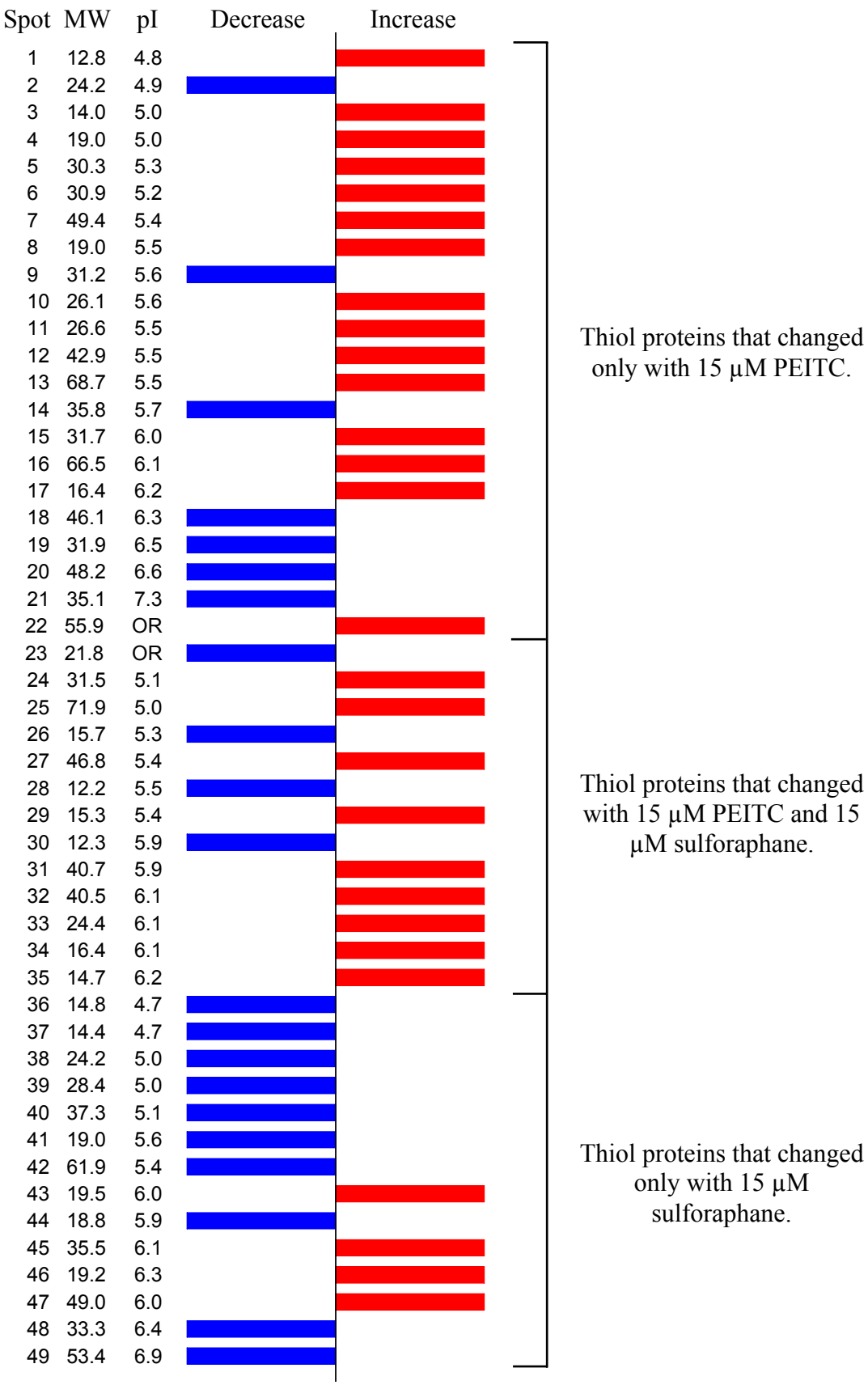


Figure 5.9: Summary of the changes in IAF labelling when B9 cells were treated with PEITC or sulforaphane. *OR=out of range*

5.4 Discussion

Previous studies investigating the mechanisms that underlie the induction of apoptosis by the isothiocyanates have focussed on the modulation of particular components of signal transduction pathways known to influence activation of apoptosis (Chen et al. 1998; Xu and Thornalley 2001b; Jeong et al. 2004; Singh et al. 2004). Although these studies have identified changes in the activities of particular pathways in response to isothiocyanate exposure, there is no evidence as to whether such alterations are a primary response, key to mediating isothiocyanate activity, or just a secondary effect, downstream of a more critical pathway. It is also more than likely that the induction of apoptosis by the isothiocyanates is not achieved by blocking or activating a single signalling pathway but, instead, via the modulation of multiple cellular events. In this study, the response of the entire cellular complement of proteins to isothiocyanate treatment was monitored. Using a fluorescence labelling technique, changes in the pattern of oxidised thiol proteins in Bcl-2 overexpressing cells, induced by isothiocyanate exposure, have been detected. While a total of 390 thiol protein spots were detected, only 49 spots consistently changed when cells were treated with PEITC or sulforaphane.

Changes in the oxidation state of protein thiol residues were observed as either an increase or decrease in the intensity of IAF labelling. The nature of the change provides some insight into the type of modification that occurs in response to isothiocyanate treatment. Oxidation of protein thiol residues can result in the formation of a number of products. These include intra- or intermolecular disulfide bonds, mixed disulfides with GSH or sulfenic acids, all of which are readily reversed by DTT. Alternatively sulfinic or sulfonic acids can be produced, but these changes are irreversible (Ghezzi 2005).

An increase in IAF labelling of a thiol protein will result from reversible oxidation in response to isothiocyanate treatment (Figure 5.10A). Alternatively, a loss of IAF labelling can result from irreversible oxidation of a cysteine residue, of which some fraction occurs as being reversibly oxidised before treatment (Figure 5.10B). A decrease in IAF labelling will also occur with a protein whose oxidation leads to a positional change in the gel.

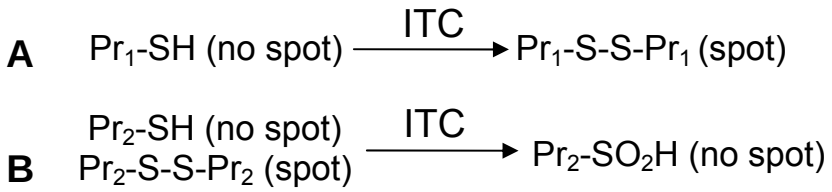


Figure 5.10: IAF labelling of protein thiol residues. ***(A)*** *If a thiol residue is reversibly oxidised in response to isothiocyanate treatment an increase in IAF labelling will be observed.* ***(B)*** *A loss of IAF labelling can result from irreversible oxidation of a cysteine residue, of which some fraction occurs as being reversibly oxidised before treatment*

Changes in the fluorescence intensity of thiol proteins may also potentially result from the thiocarbamoylation reaction between an isothiocyanate and a protein thiol residue. If the dithiocarbamate linkage is disrupted after NEM treatment, but prior to addition of IAF, an increase in fluorescence will be observed, as the isothiocyanate will block the reaction of the thiol with NEM (Figure 5.11A). If the stability of the dithiocarbamate linkage is sufficient to withstand the labelling procedure, then a decrease in IAF fluorescence will be observed in treated samples, as long as a fraction of the protein is in a reversibly oxidised state prior to treatment (Figure 5.11B). Alternatively, dithiocarbamate formation could result in a decrease in fluorescence if the thiocarbamoylation reaction were to disrupt an inter- or intramolecular disulfide linkage.

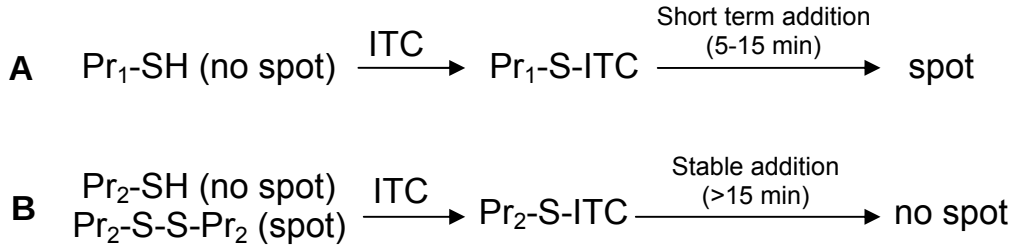


Figure 5.11: The influence of dithiocarbamate formation on IAF labelling of protein thiol residues. ***(A)*** *If the dithiocarbamate linkage is disrupted after NEM treatment but prior to addition of IAF, an increase in fluorescence will be observed.* ***(B)*** *If the stability of the dithiocarbamate linkage is sufficient to withstand the labelling procedure then a decrease in IAF fluorescence will be observed in treated samples, as long as a fraction of the protein is in a reversibly oxidised state prior to treatment*

A large majority of thiol proteins altered in response to both sulforaphane and PEITC treatment, or in response to PEITC treatment only, experienced an increase the degree

of IAF labelling (69% and 68% respectively). In contrast, only 29% of thiol proteins that were modified by exposure to sulforaphane demonstrated an increase in fluorescence with most of the spots decreasing in fluorescence (71%). The reason for this difference is not immediately apparent but it warrants further investigation.

The modification of thiol proteins that resulted from exposure to sulforaphane occurred at a concentration that is incapable of inducing B9 cell death. This suggests that the changes to thiol proteins identified in response to sulforaphane, or in response to either sulforaphane or PEITC treatment, represent potential targets that may mediate other known biological effects of the isothiocyanates, including modulation of phase I and phase II enzymatic activity. Conversely, the changes that occurred specifically in response to treatment with PEITC may be involved in the activation of apoptosis.

Two changes that were documented exclusively following exposure to PEITC were to spot number 10 and spot number 11. Based on the identification of thiol proteins sensitive to H_2O_2-induced oxidation (Baty et al. 2005), spot 10 and spot 11 appear to represent peroxiredoxin-3 (Prx-3). An increase in fluorescence labelling was observed following PEITC treatment, indicating that the cysteine residues of this protein may become oxidised as a result of exposure to PEITC, but not in response to sulforaphane treatment. Prx-3 contains two conserved cysteine residues, both of which are required for activity (Rhee et al. 2005). The peroxiredoxins, and a number of other peroxidases, protect cells against ROS-induced damage by scavenging H_2O_2. Prx-3 is localised exclusively to the mitochondrial matrix, where it is required to maintain normal mitochondrial function and where it can protect cells from oxidant-induced apoptosis (Araki et al. 1999; Wonsey et al. 2002; Nonn et al. 2003). The significance of the oxidation of Prx-3 in terms of the induction of apoptosis by PEITC remains to be elucidated, but demonstrates that proteins involved in the regulation of apoptosis are modified following a short exposure to this isothiocyanate.

Interestingly, the pattern of thiol oxidation in response to isothiocyanate treatment was very different to changes observed when Jurkat cells were treated with H_2O_2 and the thiol alkylating agent diamide or 1-chloro-2,4-dinitrobenzene (DNCB) that disrupts thiol reduction pathways (Baty et al. 2005). This suggests that the changes

observed in this investigation are not the result of general oxidative stress. Studies have suggested that Bcl-2 overexpressing cells might be more resilient to H_2O_2-induced damage (Ellerby et al. 1996); therefore, the disparity between experiments may simply reflect the differences between the cell lines utilised in each experiment. However, studies in our lab have shown that GAPDH and the peroxiredoxins are equally susceptible to H_2O_2-induced oxidation, regardless of the extent of Bcl-2 overexpression (experimental work performed by Andrew Cox). This indicates that we have been able to identify thiol proteins that are exclusively sensitive to oxidative modification in response to isothiocyanate exposure.

Chapter 6

Summary and future directions

Because the relation between cancer and deregulation of apoptosis is so strong (Martin and Green 1995; Brown and Attardi 2005), any therapeutic strategy aimed at specifically triggering apoptosis in cancer cells might have potential therapeutic efficacy. The identification of agents that are able to induce apoptosis in cancer cells, regardless of the cells' status of resistance, is now recognised as a key step in the continuing struggle against the cancer epidemic (Fesik 2005).

The isothiocyanates are emerging as a class of compounds that have potential application to both the prevention and treatment of cancer. The biological activities of the isothiocyanates include inhibition of phase I carcinogen activating enzymes and induction of phase II detoxification enzymes (Hecht 1995; Hecht 2000). Furthermore, these naturally occurring phytochemicals have been shown to inhibit cell cycle progression and induce apoptosis (Conaway et al. 2002; Zhang et al. 2006). The primary aim of this thesis was to directly examine the ability of the isothiocyanates to induce apoptosis in cells overexpressing the anti-apoptotic protein Bcl-2.

Initially, the cytotoxic activity of PEITC was examined. PEITC rapidly inhibited proliferation of Jurkat cells within 1 hour. At low concentrations, this was a temporary growth arrest; however, at higher concentrations (greater than 5 μM) caspase activation was observed after 2 hours, leading to apoptotic cell death. At the highest concentrations tested (40 μM to 60 μM) no caspase activation was observed, but the cells died by necrosis. It was also demonstrated that PEITC was able to overcome the anti-apoptotic block that overexpression of Bcl-2 confers. The presence of Bcl-2 expression had very little effect on the cytotoxicity of PEITC, with only a shifting of the dose-response curve at the highest levels of Bcl-2 expression. Furthermore, the isothiocyanates were still able to trigger apoptosis in these cells.

The key structural features of the isothiocyanates that confer an ability to overcome Bcl-2 overexpression were also identified. Of the sixteen isothiocyanates screened in

this study, PEITC was identified as the most promising compound for targeting Bcl-2 positive cells. While the isothiocyanate moiety ultimately determined cytotoxic activity, the chemistry of the side chain played a key role in the ability of an isothiocyanate to kill Bcl-2 overexpressing cells. The aromatic isothiocyanates were generally far more efficacious than aliphatic isothiocyanates; however, minor structural changes between the aromatic isothiocyanates significantly reduced cytotoxic activity. The requirement for rigid structural features suggests that the isothiocyanates may specifically interact with cellular targets that are involved in activation of apoptosis.

The other important property of the aromatic isothiocyanates is that they caused a complete loss of cell viability, in contrast to the phenomenon observed with standard chemotherapeutic agents, including melphalan. Complete loss of cancer cell viability is important. If any cells within a transformed population are able to resist a cytotoxic compound, they will continue to proliferate and reform a more resistant population. Results from this *in vitro* study suggest that such a phenomenon would not occur following exposure to PEITC. However, many non-aromatic isothiocyanates investigated in this study were only capable of killing a proportion of Bcl-2 overexpressing cells, and at much higher concentrations than PEITC required.

The final aim of the thesis was to identify the molecular targets of the aromatic isothiocyanates that enable these compounds to overcome the anti-apoptotic function of Bcl-2. Fluorescent labelling of modified thiol proteins combined with 2-D electrophoresis enabled a preliminary investigation of some of the targets of the isothiocyanates. A number of protein thiols were selectively modified when Bcl-2 overexpressing cells were exposed to PEITC. The modifications were observed following only thirty minutes exposure to PEITC, indicating that such changes may play a role in mediating activation of apoptosis. One such modified thiol protein was identified as peroxiredoxin-3. As yet the significance of this modification is unknown.

The ability of PEITC to induce apoptosis and loss of cell viability in cells that overexpress Bcl-2 suggests that this compound can either activate an alternate route to bypass the apoptotic block that overexpression of Bcl-2 confers, or directly interfere with the anti-apoptotic properties of Bcl-2.

A potential explanation for the inability of Bcl-2 to prevent apoptosis is that the PEITC may induce apoptosis via the receptor-mediated apoptotic pathway. Modification of proteins that are associated with the DISC, and subsequent promotion of caspase-8 activation, has been suggested as a mechanism by which isothiocyanates trigger apoptosis (Thornalley 2002). However, Jurkat cells are type II cells (Cuvillier et al. 2000) and overexpression of Bcl-2 blocks the receptor-mediated pathway in our cell system. In direct contrast to this hypothesis, many studies support the induction of the mitochondria-dependent apoptotic pathway, characterised by activation of caspase-9, in response to isothiocyanate exposure (Nakamura et al. 2002; Hu et al. 2003; Gingras et al. 2004; Rose et al. 2005; Zhang et al. 2005)). Several isothiocyanates have been shown to cause direct perturbations to the mitochondrial membrane, which induce the release of cytochrome c and allow for the subsequent activation of caspase-9 (Rose et al. 2005; Wu et al. 2005; Zhang et al. 2005). By directly affecting the permeability of the mitochondrial membrane, the isothiocyanates may be able to overcome Bcl-2 overexpression by directly bypassing this apoptotic block.

While the isothiocyanates may induce apoptosis via a mechanism that is not reliant on the expression of Bcl-2, a large collection of evidence supports a direct role for members of the Bcl-2 family in isothiocyanate-induced apoptosis. Several studies have demonstrated upregulation of the expression of pro-apoptotic Bcl-2 family members, in particular Bax (Gamet-Payrastre et al. 2000; Fimognari et al. 2004b; Srivastava and Singh 2004; Wu et al. 2005; Xiao et al. 2005b). The upregulation of Bax that occurs in response to isothiocyanate exposure may be sufficient to push the equilibrium between pro- and anti-apoptotic factors towards favouring the induction of apoptosis. Furthermore, the isothiocyanates have been shown to induce the activation and translocation of Bax to the mitochondrial membrane and also to prevent the heterodimerization of Bcl-2 with Bax at the mitochondrial membrane (Miyoshi et al. 2004b; Rose et al. 2005; Zhang et al. 2005). A key role for pro-apoptotic Bcl-2 family members in the induction of apoptosis by isothiocyanates has been further implicated as a result of studies with Bax and Bak double knockout mice, which are significantly more resistant to the cytotoxic activity of PEITC (Xiao et al. 2005b). Interestingly, *in vitro* and *in vivo* evidence has demonstrated down-regulation of Bcl-2 expression in response to treatment with isothiocyanates (Srivastava et al.

2003; Singh et al. 2004; Srivastava and Singh 2004; Wu et al. 2005), although this is a late event and, therefore, unlikely to explain the results obtained in this study

Although potential targets of PEITC, that may play a role in triggering apoptosis, have been identified in this research, the protocol probably identified thiol protein targets that are indirectly modified in response to isothiocyanate exposure. Given that a key reaction of intracellular isothiocyanates is conjugation with protein thiols to form dithiocarbamates, it seems reasonable to hypothesise that such thiocarbamoylation reactions may play a key role in isothiocyanate-induced apoptosis. If dithiocarbamate formation is crucial to the ability of PEITC to induce apoptosis in cells that overexpress Bcl-2, a technique to identify the direct formation of a dithiocarbamate between PEITC and a thiol protein target must be developed.

Due to the instability of the dithiocarbamate linkage, supporting evidence for the conjugation of an isothiocyanate with cysteinyl thiol groups is sparse. A direct interaction of benzyl isothiocyanate with Cys25 of papain was hypothesised to be key in isothiocyanate-mediated inhibition of enzymatic activity, although no direct evidence was available (Tang and Tang 1976). A recent study with Keap1 clearly demonstrated the modification of thiol residues by directly monitoring adduct formation with sulforaphane (Hong et al. 2005). By regulating the activity of the ARE-binding transcription factor Nrf-2, Keap-1 prevents the induction of phase II enzymes and antioxidant enzymes that play a key role in chemoprevention (Itoh et al. 1999). Modification of Keap1 by isothiocyanates therefore leads to transcription of ARE-regulated genes, which explains the potent induction of phase II enzymes in response to isothiocyanate treatment. Furthermore, isothiocyanate-induced mitochondrial damage and cytochrome c release have recently been hypothesised to result from an ITC-moiety-dependent reaction with critical thiol groups in the adenine nucleotide translocase, which forms part of the mitochondrial permeability transition pore (Kawakami et al. 2005).

The need to monitor the formation of dithiocarbamates becomes even more evident when considering a potential mechanism to explain the ability of PEITC to induce apoptosis, regardless of the extent of Bcl-2 overexpression. On the basis of this research it is hypothesised that PEITC may directly bind to, and adversely affect, the

anti-apoptotic function of Bcl-2. The primary structure of Bcl-2 contains two cysteine residues, Cys158 and Cys229 which lie in the α5 helix and the transmembrane domain respectively (Petros et al. 2004) (section 1.3.2). Whereas Cys229 is largely sequestered due to the membrane localisation of Bcl-2, Cys158 is free to react with thiol-reactive compounds. Although Cys158 is removed from the hydrophobic binding groove of Bcl-2, and does not play a role in heterodimerization between Bcl-2 and pro-apoptotic family members, disruption of this residue has been shown to reduce the ability of Bcl-2 to prevent apoptosis (Maser et al. 2000). Binding of PEITC to Cys158 may, therefore, at least partially abrogate the anti-apoptotic function of Bcl-2 and allow progression of the apoptotic pathway following isothiocyanate exposure.

Site-directed mutagenesis of the cysteine residues in Bcl-2 would allow for direct insight into the importance of dithiocarbamate formation between PEITC and Bcl-2 and the ability to overcome overexpression of Bcl-2. This technique could also be applied to peroxiredoxin 3, to gain an insight into the importance of oxidation of this thiol protein in the activation of PEITC-induced apoptosis. If the isothiocyanates do interact with thiol residue(s) of proteins such as Bcl-2, the rigid structural features that govern cytotoxicity in Bcl-2 overexpressing cells might become clearer. Furthermore, if Bcl-2 or any other apoptosis-regulatory proteins are identified as targets of PEITC, the structural features of the compound can be optimised to maximise induction of apoptosis in drug-resistant cells.

Although this study has identified that some isothiocyanates possess an ability to induce apoptotic cell death, it is also crucial to identify whether the isothiocyanates are selectively toxic to cancerous cells. Selective targeting and low toxicity for normal tissues are fundamental requisites for proposed chemopreventive agents. If the isothiocyanates are to be applied in a clinical setting, it would be advantageous if populations of non-transformed cells could remain untouched, while transformed cells are eliminated by the compound. Preliminary studies have demonstrated that PEITC, allyl isothiocyanate and 4-(methylthio)butyl isothiocyanate are significantly more toxic to transformed cells (Musk and Johnson 1993; Adesida et al. 1996; Xu and Thornalley 2000b; Fimognari et al. 2004a; Fimognari et al. 2004c; Xiao et al. 2005). In contrast, sulforaphane displays selectivity for colon carcinoma cells but adversely

affects the viability of both non-transformed T lymphocytes and Jurkat cells (Fimognari et al. 2002; Fimognari et al. 2004a). While most of these studies have investigated selectivity in cultured cell systems, it would be beneficial to investigate transformed and non-transformed cells taken directly from cancer patients.

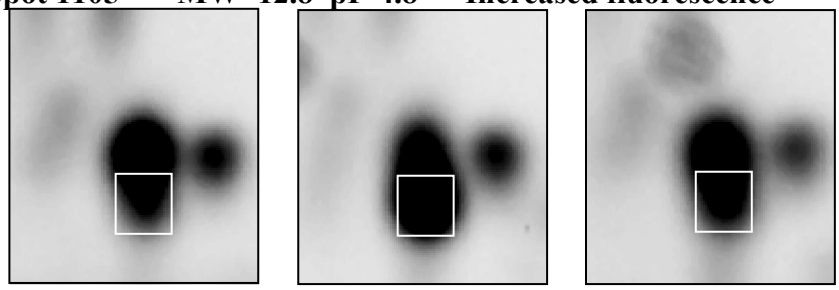
In summary, the results of this thesis have provided a platform to permit further investigation of the chemotherapeutic potential of the isothiocyanates and investigation of the mechanisms that allow the isothiocyanates, in particular PEITC, to induce apoptosis in cells that overexpress the oncogene Bcl-2. In the future, the identification of primary targets of the isothiocyanates will form the basis for drug design and will also provide a novel insight into the regulation of apoptosis.

Appendix

Enlarged images of individual spot changes identified in Chapter 5

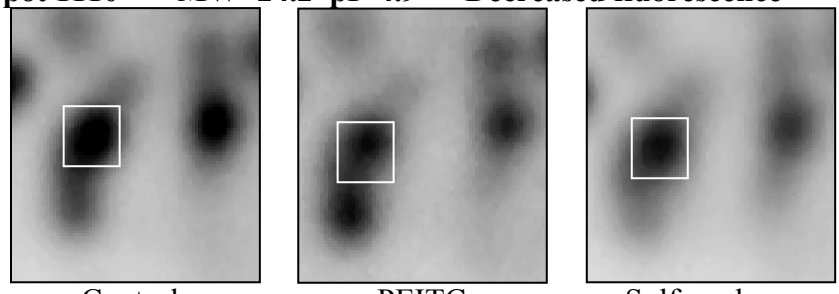
Changes to the intensity of IAF labelling of individual proteins were analysed with PDQuest™ 2-D electrophoresis gel analysis software (version 7.1.1, Bio-Rad Laboratories, Hercules, CA, USA). Thiol proteins with a two-fold increase or decrease in IAF fluorescence in response to isothiocyanate exposure were selected. Only thiol proteins that consistently changed in at least two of the three experiments are shown. Spot numbering is consistent with highlighted spots in Figure 5.6 and Figure 5.8. Squares indicate the position of thiol proteins that changed. An estimation of molecular weight (MW) and isoelectric point (pI) values for each spot was made by comparison with the positions of proteins with known MW and pI values.

1 Spot 1105 MW=12.8 pI=4.8 Increased fluorescence



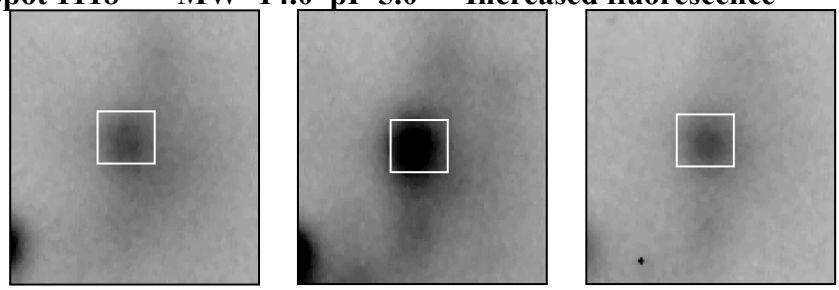
Control PEITC Sulforaphane

2 Spot 1110 MW=24.2 pI=4.9 Decreased fluorescence



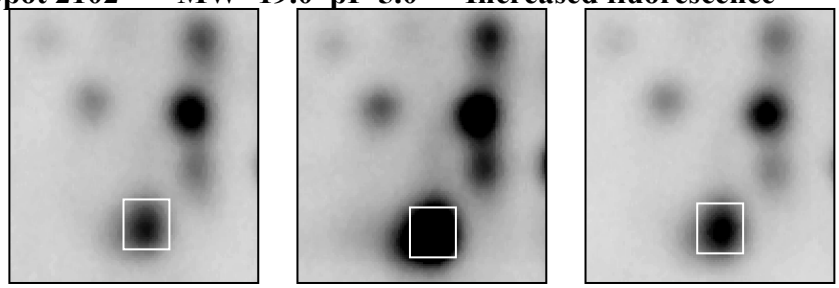
Control PEITC Sulforaphane

3 Spot 1118 MW=14.0 pI=5.0 Increased fluorescence



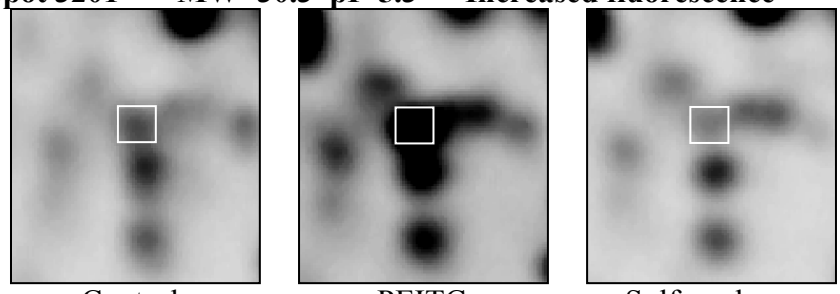
Control PEITC Sulforaphane

4 Spot 2102 MW=19.0 pI=5.0 Increased fluorescence



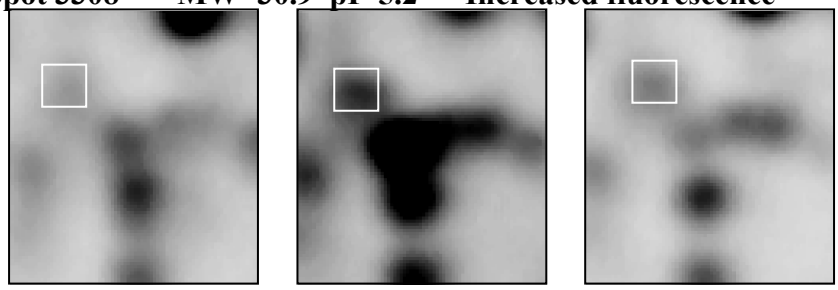
Control PEITC Sulforaphane

5 Spot 3201 MW=30.3 pI=5.3 Increased fluorescence



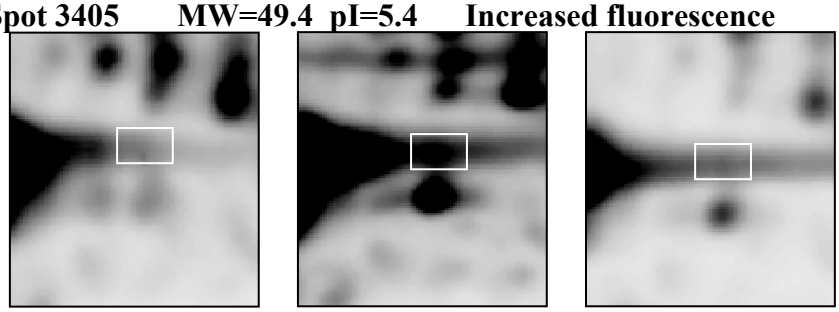
Control PEITC Sulforaphane

6 Spot 3308 MW=30.9 pI=5.2 Increased fluorescence



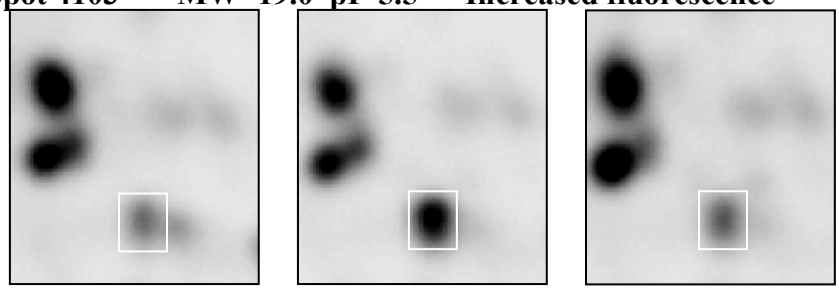
Control PEITC Sulforaphane

7 Spot 3405 MW=49.4 pI=5.4 Increased fluorescence



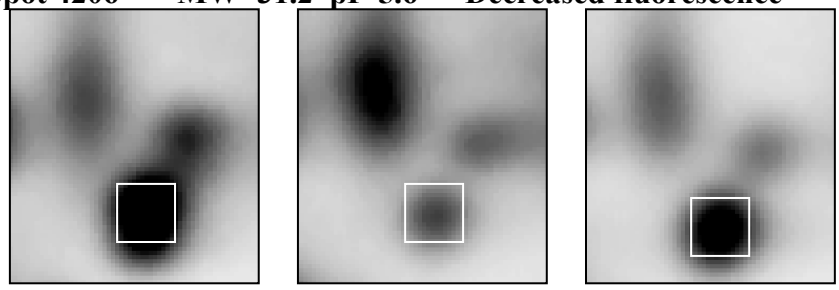
Control PEITC Sulforaphane

8 Spot 4103 MW=19.0 pI=5.5 Increased fluorescence



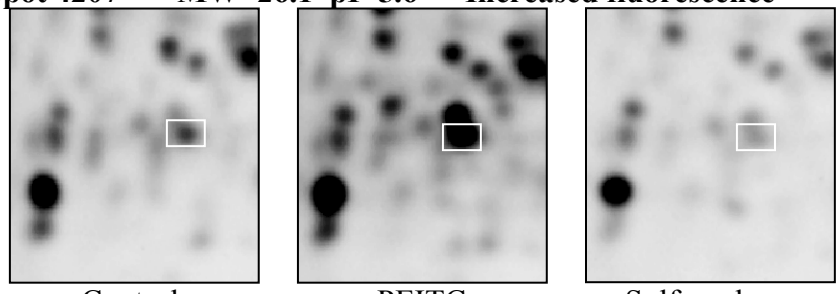
Control PEITC Sulforaphane

9 Spot 4206 MW=31.2 pI=5.6 Decreased fluorescence



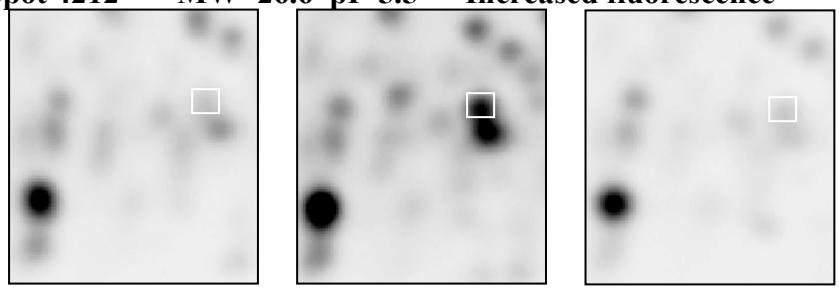
Control PEITC Sulforaphane

10 Spot 4207 MW=26.1 pI=5.6 Increased fluorescence



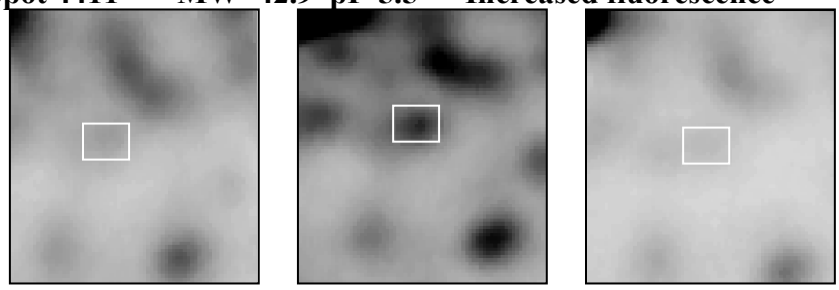
Control PEITC Sulforaphane

11 Spot 4212 MW=26.6 pI=5.5 Increased fluorescence



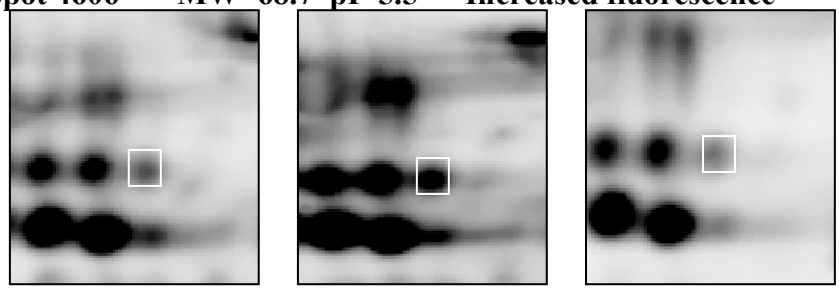
Control PEITC Sulforaphane

12 Spot 4411 MW=42.9 pI=5.5 Increased fluorescence



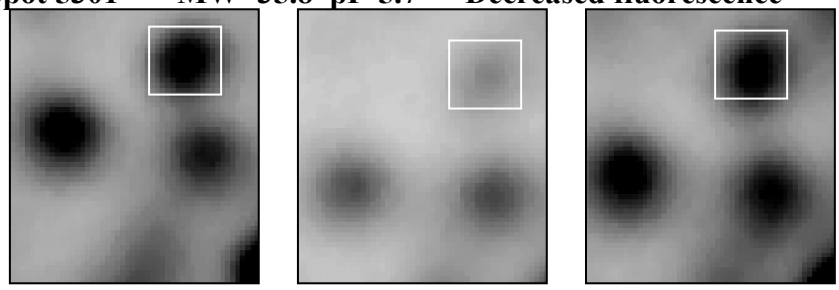
Control PEITC Sulforaphane

13 **Spot 4606 MW=68.7 pI=5.5 Increased fluorescence**



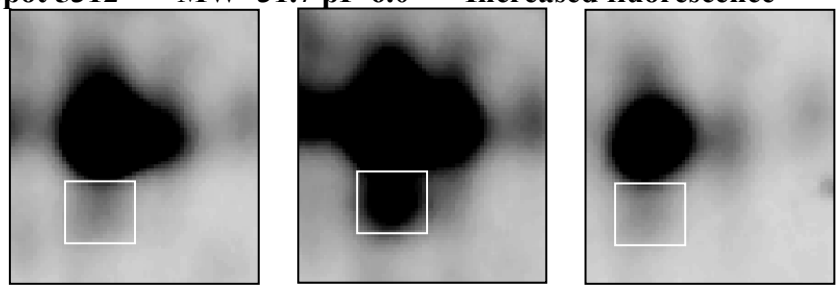
Control PEITC Sulforaphane

14 **Spot 5301 MW=35.8 pI=5.7 Decreased fluorescence**



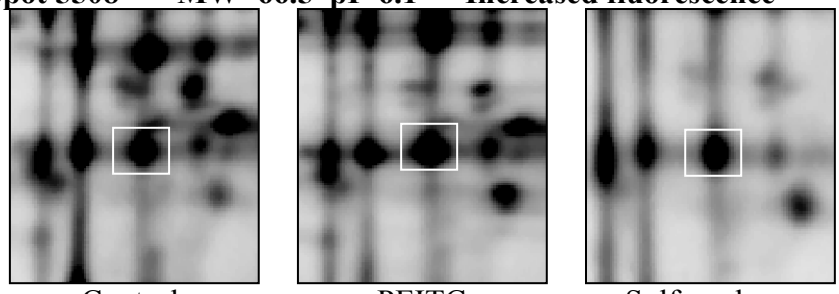
Control PEITC Sulforaphane

15 **Spot 5312 MW=31.7 pI=6.0 Increased fluorescence**



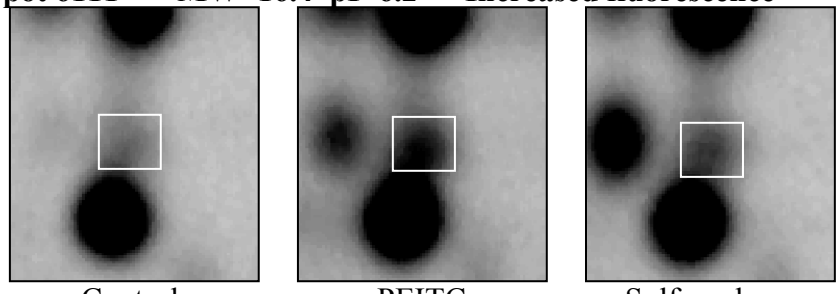
Control PEITC Sulforaphane

16 **Spot 5508 MW=66.5 pI=6.1 Increased fluorescence**



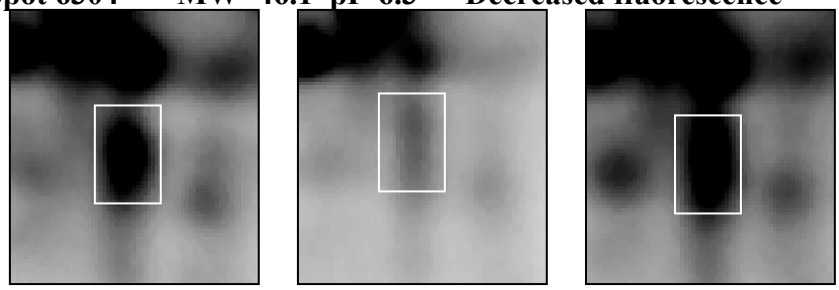
Control PEITC Sulforaphane

17 **Spot 6111 MW=16.4 pI=6.2 Increased fluorescence**



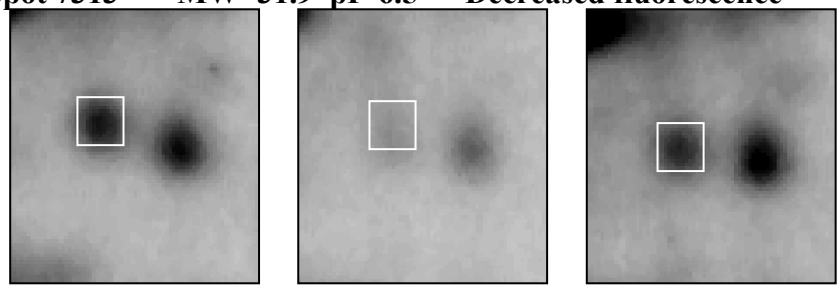
Control PEITC Sulforaphane

18 Spot 6304 MW=46.1 pI=6.3 Decreased fluorescence



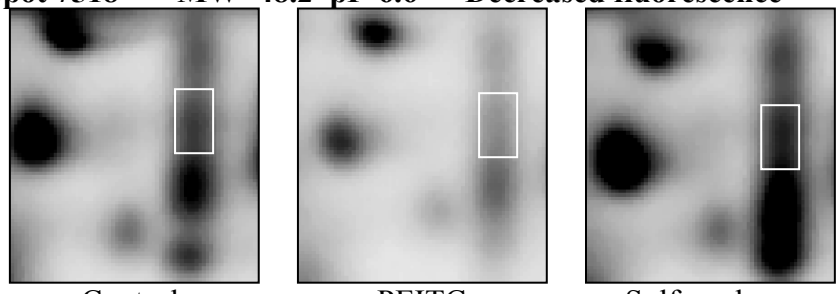
Control PEITC Sulforaphane

19 Spot 7313 MW=31.9 pI=6.5 Decreased fluorescence



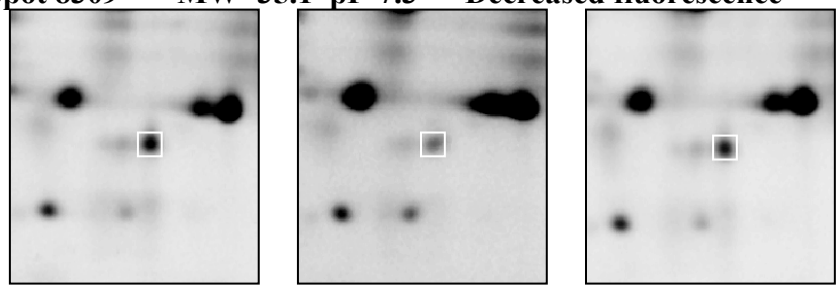
Control PEITC Sulforaphane

20 Spot 7518 MW=48.2 pI=6.6 Decreased fluorescence



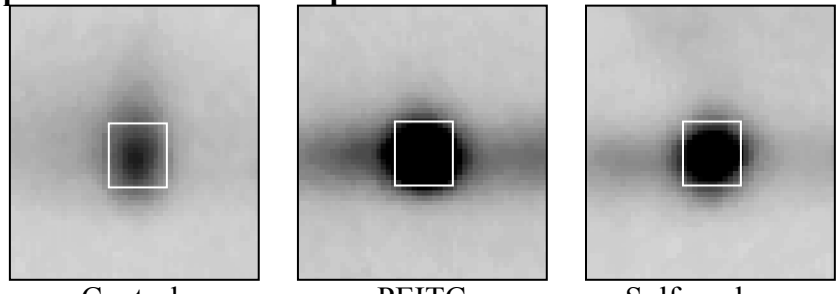
Control PEITC Sulforaphane

21 Spot 8309 MW=35.1 pI=7.3 Decreased fluorescence



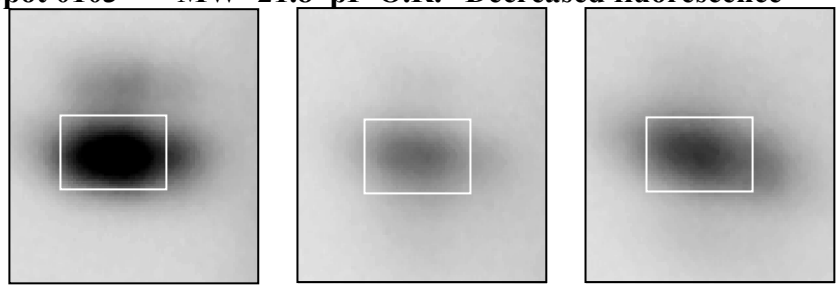
Control PEITC Sulforaphane

22 Spot 8405 MW=55.9 pI=O.R. Increased fluorescence



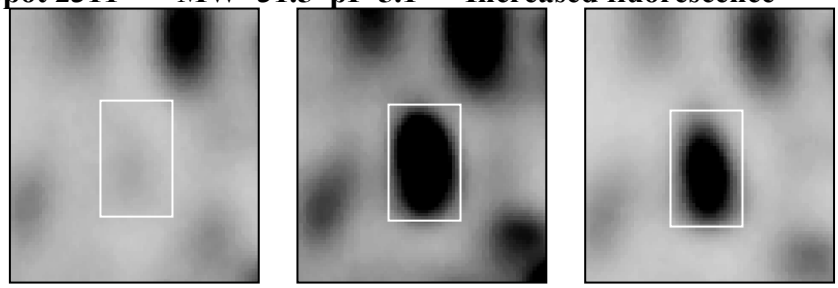
Control PEITC Sulforaphane

23 Spot 0103 MW=21.8 pI=O.R. Decreased fluorescence



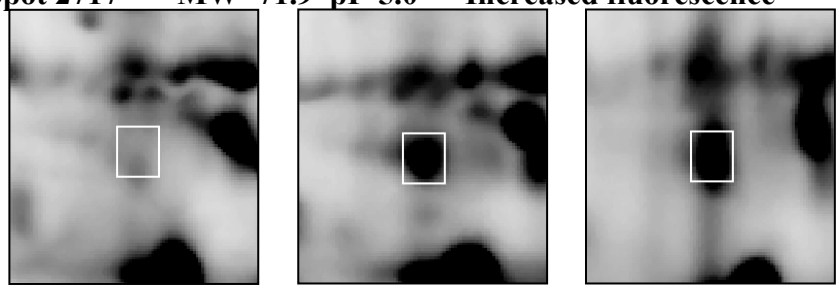
Control PEITC Sulforaphane

24 Spot 2311 MW=31.5 pI=5.1 Increased fluorescence



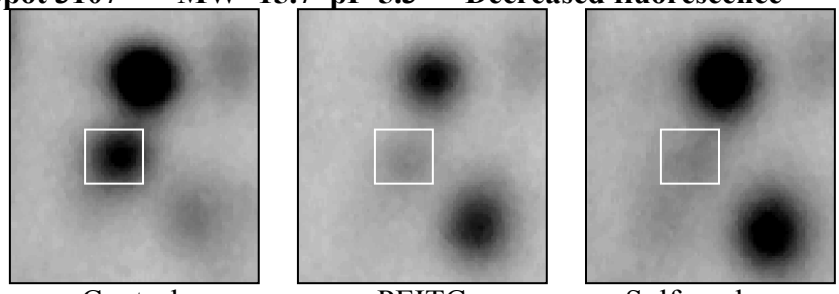
Control PEITC Sulforaphane

25 Spot 2717 MW=71.9 pI=5.0 Increased fluorescence



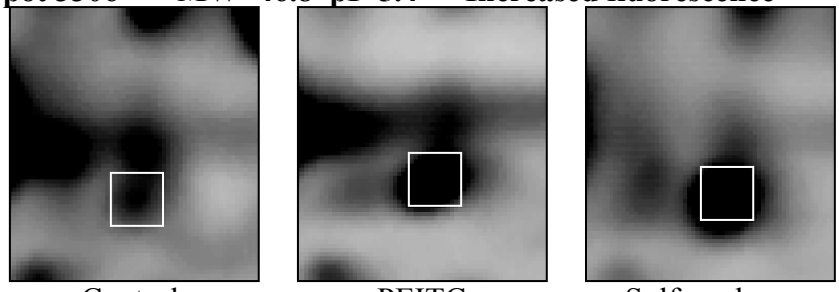
Control PEITC Sulforaphane

26 Spot 3107 MW=15.7 pI=5.3 Decreased fluorescence



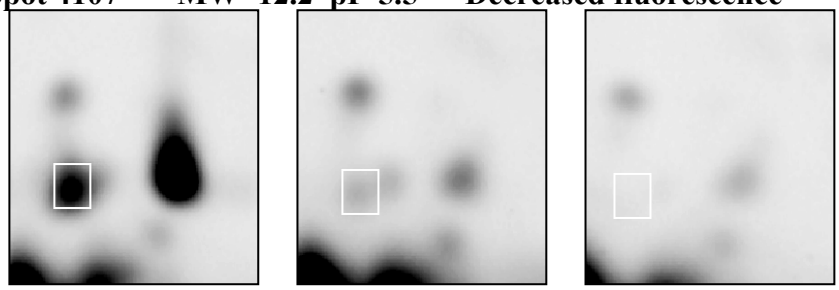
Control PEITC Sulforaphane

27 Spot 3306 MW=46.8 pI=5.4 Increased fluorescence



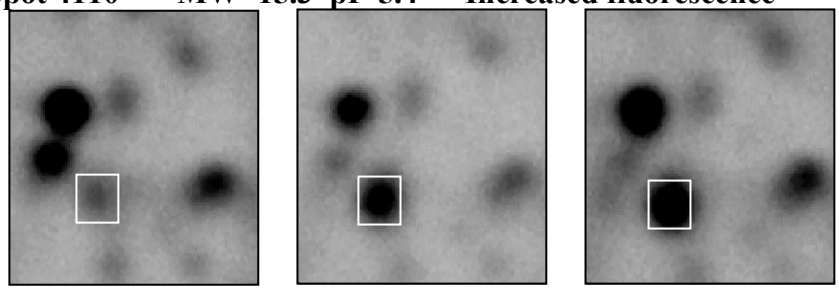
Control PEITC Sulforaphane

28 Spot 4107 MW=12.2 pI=5.5 Decreased fluorescence



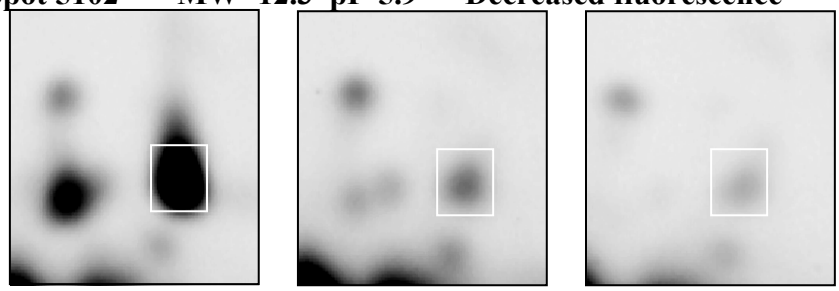
Control PEITC Sulforaphane

29 Spot 4110 MW=15.3 pI=5.4 Increased fluorescence



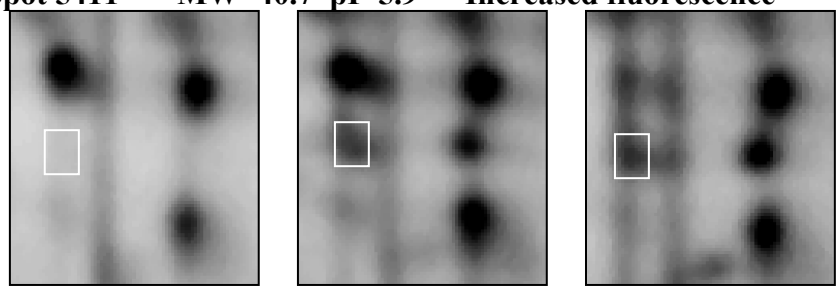
Control PEITC Sulforaphane

30 Spot 5102 MW=12.3 pI=5.9 Decreased fluorescence



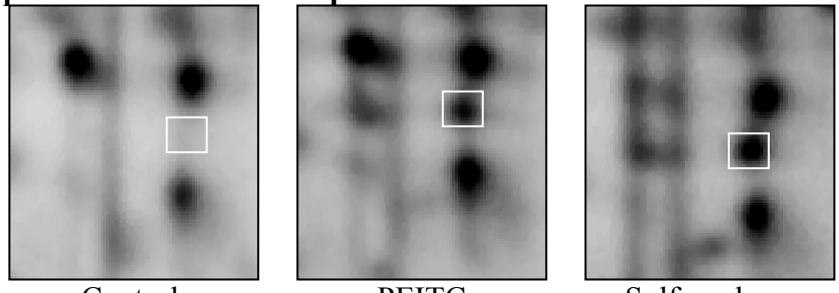
Control PEITC Sulforaphane

31 Spot 5411 MW=40.7 pI=5.9 Increased fluorescence



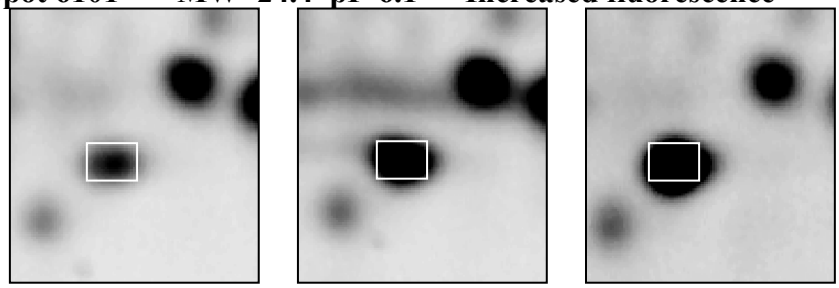
Control PEITC Sulforaphane

32 Spot 5412 MW=40.5 pI=6.1 Increased fluorescence



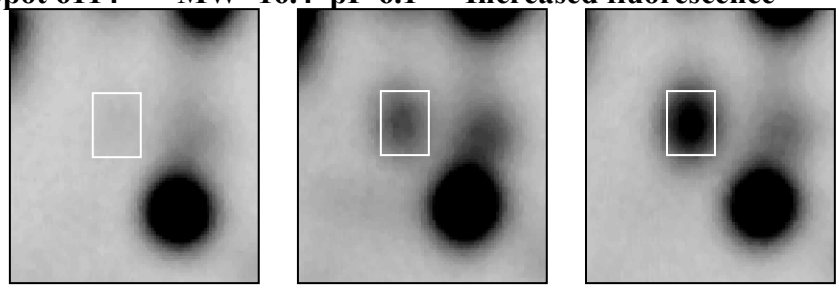
Control PEITC Sulforaphane

33 **Spot 6101 MW=24.4 pI=6.1 Increased fluorescence**



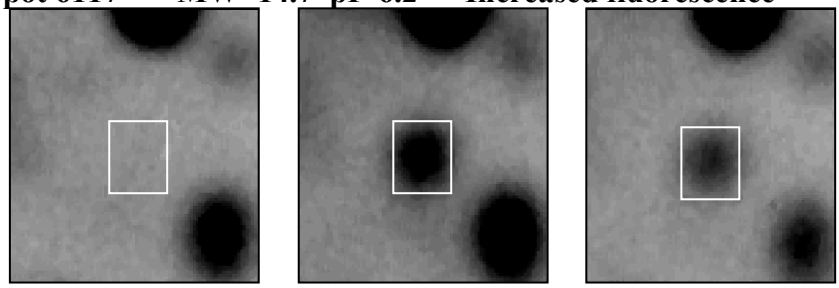
Control PEITC Sulforaphane

34 **Spot 6114 MW=16.4 pI=6.1 Increased fluorescence**



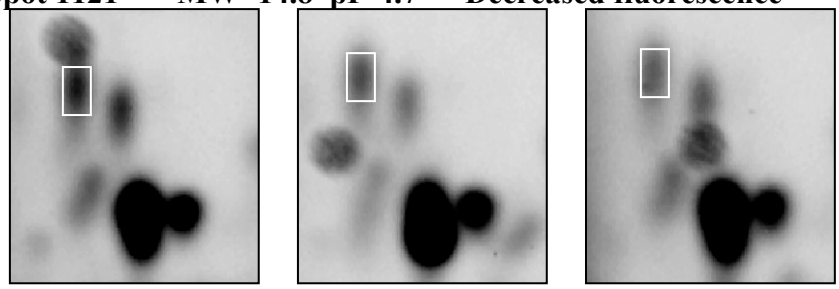
Control PEITC Sulforaphane

35 **Spot 6117 MW=14.7 pI=6.2 Increased fluorescence**



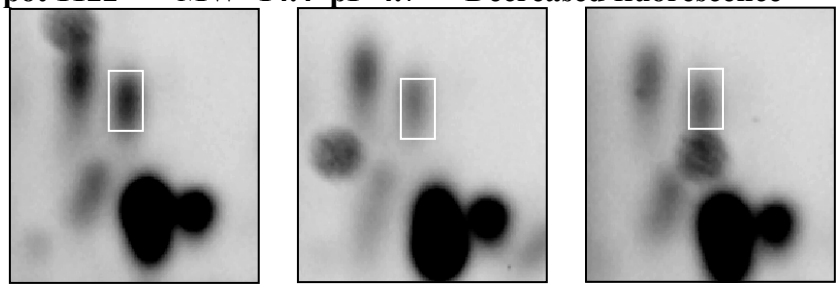
Control PEITC Sulforaphane

36 **Spot 1121 MW=14.8 pI=4.7 Decreased fluorescence**



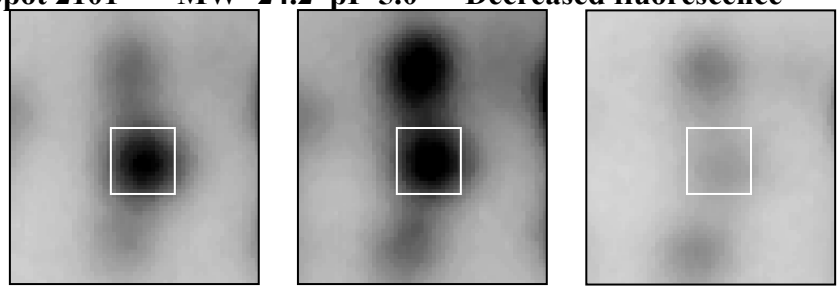
Control PEITC Sulforaphane

37 **Spot 1122 MW=14.4 pI=4.7 Decreased fluorescence**



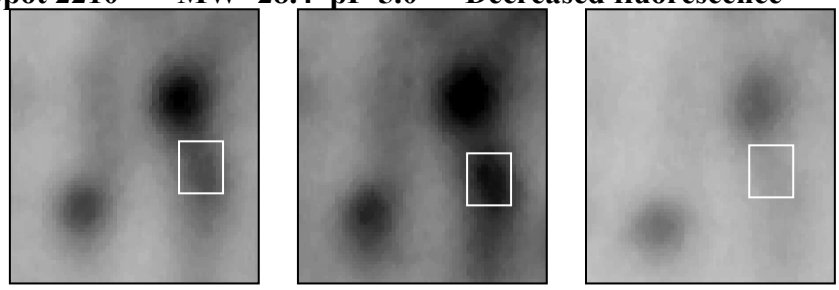
Control PEITC Sulforaphane

38 Spot 2101 MW=24.2 pI=5.0 Decreased fluorescence



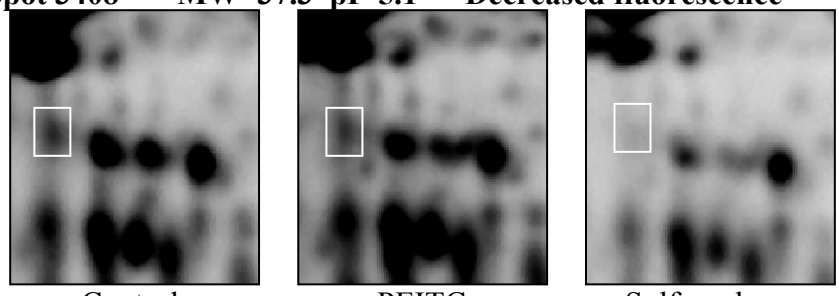
Control PEITC Sulforaphane

39 Spot 2210 MW=28.4 pI=5.0 Decreased fluorescence



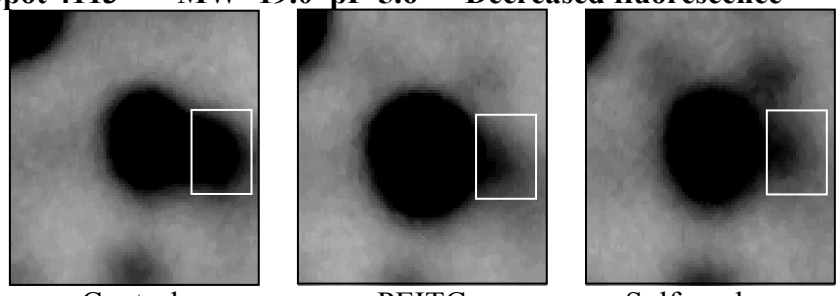
Control PEITC Sulforaphane

40 Spot 3408 MW=37.3 pI=5.1 Decreased fluorescence



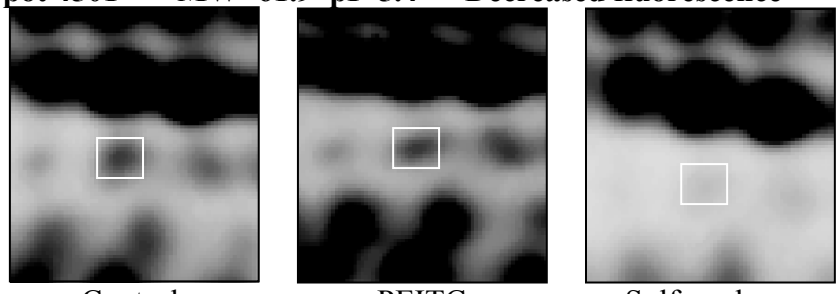
Control PEITC Sulforaphane

41 Spot 4113 MW=19.0 pI=5.6 Decreased fluorescence



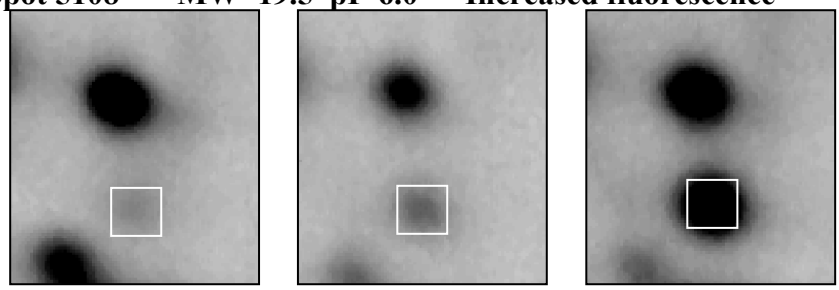
Control PEITC Sulforaphane

42 Spot 4501 MW=61.9 pI=5.4 Decreased fluorescence



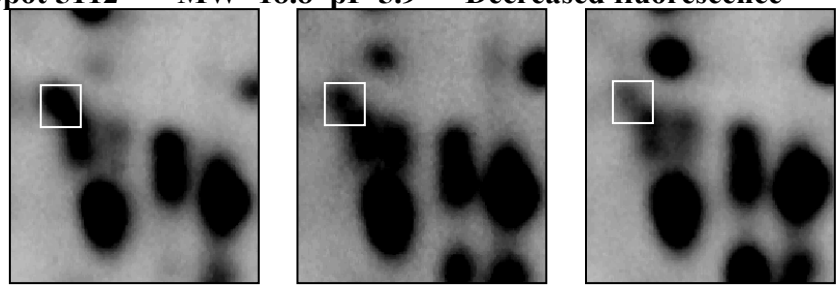
Control PEITC Sulforaphane

43 Spot 5108 MW=19.5 pI=6.0 Increased fluorescence



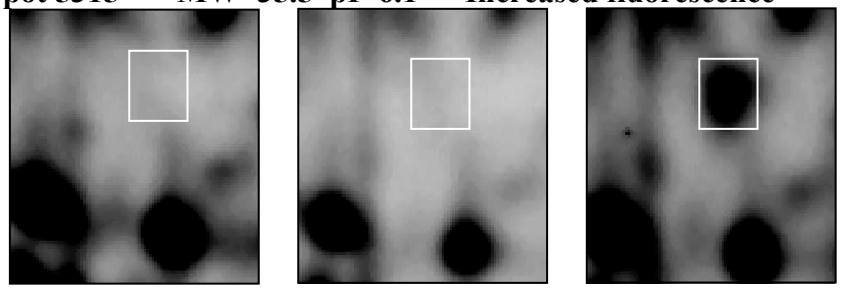
Control PEITC Sulforaphane

44 Spot 5112 MW=18.8 pI=5.9 Decreased fluorescence



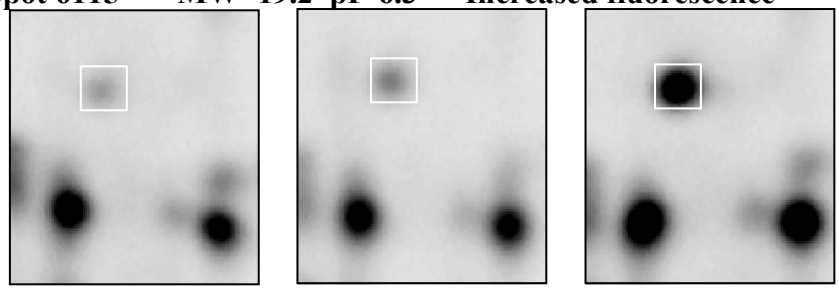
Control PEITC Sulforaphane

45 Spot 5315 MW=35.5 pI=6.1 Increased fluorescence



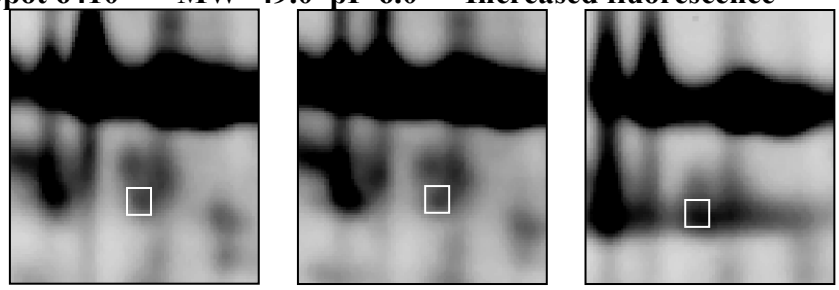
Control PEITC Sulforaphane

46 Spot 6115 MW=19.2 pI=6.3 Increased fluorescence



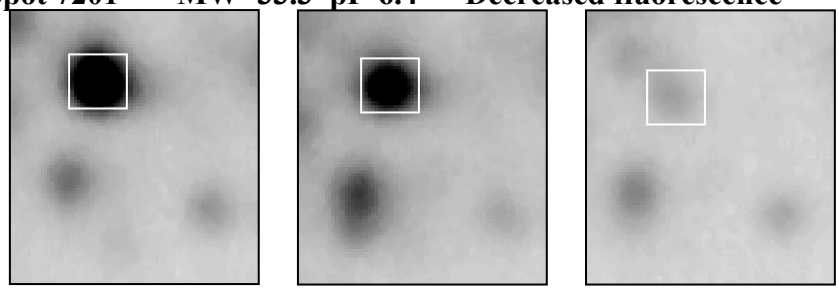
Control PEITC Sulforaphane

47 Spot 6410 MW=49.0 pI=6.0 Increased fluorescence



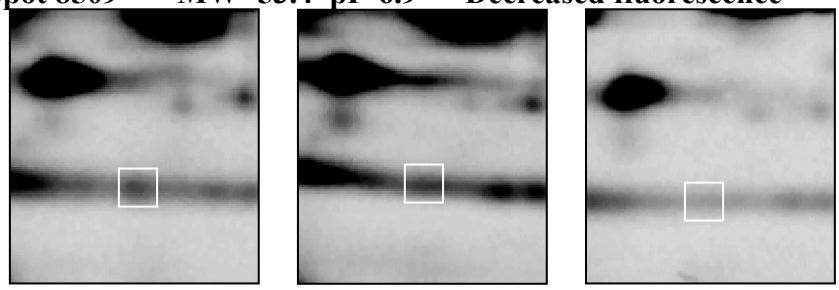
Control PEITC Sulforaphane

48 Spot 7201 MW=33.3 pI=6.4 Decreased fluorescence



Control PEITC Sulforaphane

49 Spot 8509 MW=53.4 pI=6.9 Decreased fluorescence



Control PEITC Sulforaphane

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